THE EQUINE GnRH RECEPTOR: A STUDY IN RECEPTOR STRUCTURE, FUNCTION, AND PHYSIOLOGIC BEHAVIOR

By

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Abstract of Dissertation Presented to the Graduate School
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The following experiments were designed to investigate the physiologic mechanisms that might explain the insensitivity of equine gonadotropes to constant GnRH.

The first experiments (Chapter 2) involved cloning and sequencing the equine GnRH receptor for comparison with that of other species. The cloned product represented the entire open reading frame of the equine GnRH receptor and consisted of 1158 nucleotide sequences. The sequences coded for 328 amino acids which were >85% homologous with sequences in other species. Although there existed a high degree of homology between equine GnRH receptors and other mammalian receptors, several amino acid residues were unique to the horse GnRH receptor.

The second set of experiments (Chapter 3) evaluated equine GnRH receptor binding kinetics. Data from these studies indicated that receptor affinity and number did

not differ between equids and other species. However, receptor internalization experiments demonstrated slower rates of internalization and accelerated rates of recycling for the equine GnRH receptor compared to other species. These findings were supported by electron microscopy studies, which indicated that equine GnRH receptors were observed at the cell surface following internalization of receptors inside the cell. Moreover, there was evidence suggesting that a population of receptors remained bound to the cell surface during GnRH treatment.

In the third set of experiments (Chapter 4), the steroidal regulation of the equine GnRH receptor was studied. Both ovariectomized and intact horses were used to evaluate the effects of exogenous and endogenous steroids on expression of the GnRH receptor. Data from ovariectomized mares indicated that treatment with progesterone, followed by estrogen, resulted in a 10-fold increase in receptor affinity and increased surface binding yet a decline in receptor mRNA concentrations. Additionally, mRNA from mares in estrus revealed an upregulation of GnRH receptor gene expression compared with diestrus. Furthermore, as the time of ovulation neared there appeared to be an even greater increase in GnRH receptor number. These findings indicate that estrogen has a positive feedback on the GnRH receptor.

In summary, the equine GnRH receptor does not differ significantly in structure from other species however, decreased internalization and increased recycling rates provide a mechanism for maintaining adequate concentrations of cell surface receptors. Additionally, estrogen may enhance this by upregulating gene expression during estrus.

CHAPTER 1
LITERATURE REVIEW

Gonadotropin-releasing hormone (GnRH) was isolated and structurally characterized thirty years ago using porcine, ovine, and bovine hypothalamic extracts (Amoss MR et al., 1971); (Matsuo H et al., 1971). Initially, it was termed Luteinizing hormone releasing-hormone (LHRH) because of its stimulatory effect on luteinizing hormone (LH) release. However, shortly thereafter its effect on follicle-stimulating hormone (FSH) was observed and it became known as GnRH. One of several releasing factors produced in the hypothalamus, GnRH stimulates LH and FSH release from the anterior pituitary.

GnRH Structure

The gene encoding for GnRH is located on the short (P) arm of chromosome 8 in rodents and humans (Yang-Feng TL et al., 1986). Transcription of the gene yields an mRNA strand with 4 exons, the first exon includes the 5'-untranslated region, which is tissue specific for the source of GnRH. The second exon encodes for the signal peptide, the active form of GnRH, and part of the GnRH-associated protein (GAP). The remaining two exons encode for the rest of GAP. Post-translational processing of the precursor molecule, pro-GnRH, by specific proteases results in the active form of GnRH and GAP.

Several different forms of GnRH have been discovered. At least seven forms have been isolated from various species including: mammalian GnRH, chicken GnRH, salmon GnRH, lamprey GnRH, dogfish GnRH and catfish GnRH (Sherwood NM et al., 1986). All of the identified forms of GnRH have a homologous receptor-binding domain.

The amino acid sequence of mammalian GnRH is

Pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide

GnRH Neuron Localization

Immunocytochemistry has been used to discern the innervation of GnRH-producing neurosecretory cells. The neurosecretory cells, which synthesize GnRH, release the decapeptide from stored secretory granules at the nerve terminals into the portal vasculature within the median eminence. Once in the portal vessels GnRH is transported from the median eminence to the anterior pituitary gland. As such, GnRH is a neurochemical link between the brain and the pituitary-gonadal axis. Some GnRH neurons have been shown to contain Galanin, a neurotransmitter, in the nerve terminals. Co-secretion of Galanin with GnRH may play an important role in enhancing GnRH secretion and actions. It is proposed that Galanin may interact with GnRH at the level of the anterior pituitary to increase its releasing ability of gonadotropins (LH & FSH) (Merchenthaler I et al., 1990).

The GnRH neuronal highway differs in distribution of nuclei across species. In rodents, GnRH neurons originate primarily from the septo-preoptic region. However, in primates and humans, GnRH neurons are localized in the medial hypothalamus and median eminence. The hypothalamus is divided into two major subdivisions: the medial area and the lateral area. The lateral hypothalamic region forms the lateral border of the entire medial hypothalamic nuclear complex and serves as the passageway of all medial hypothalamic nuclei to the rest of the brain. The medial hypothalamic region is heavily populated with a variety of nerve cells. In addition, a subdivision of the medial region known as the tuberal region contains most of the neurons producing hypothalamic hormones. The tuberal region includes the ventromedial nucleus, arcuate nucleus, dorsal-medial nucleus, and is closely associated with the infundibulum. Areas containing the majority of GnRH neurons within the medial hypothalamus include the organum vasculosum of the lamina terminalis (OVLT), the interstitial nucleus of the stria terminalis, the preoptic area, the periventricular nucleus, the medial septum, the diagonal band of broca, the suprachiasmatic nuclei and the arcuate nucleus (Silverman AJ et al., 1977); (Setalo GS et al., 1975). In ponies, the majorities of GnRH neurons are located within the medial basal hypothalamus and are organized in a region extending from the medial septum to tuberoinfundibular areas (Melrose PA et al., 1994). Axons from the GnRH neurons project to various sites within the brain. The key projections involving regulation of reproductive cycles include those from the medial hypothalamus to the median eminence (ventral infundibulum). Studies in ponies (Strauss SS et al., 1979), rats and sheep (Domanski E et al., 1980) have localized the median eminence as a site of high GnRH activity. The release of GnRH at the median eminence allows passage through the basal lamina and into the portal vasculature.

Small proportions of GnRH neurons originate from the posterior hypothalamus but do not terminate at the median eminence. Rather, these GnRH neurons reach the diencephalic, telencephalic and lower brain stem regions. Their roles remain unclear, however, there is evidence suggesting that GnRH may be involved in stimulating sexual behavior in rams (Schanbacker BD and Winstra DD, 1977), rodents (Moss RL and McCann SM, 1973) and geldings (Pozor MA et al., 1991). Furthermore, GnRH may act as a neurotransmitter and/or neuromodulator in processes yet to be discovered.

In addition to mammalian GnRH, non-mammalian GnRH (chicken II GnRH) has been described in the mid-brain of humans. Moreover, several studies indicated that the non-mammalian GnRH was effective in releasing LH and FSH from the mammalian pituitary, however mammalian GnRH remained a much more potent stimulator of gonadotropin secretion. Regardless, the fact that chicken II GnRH has been conserved in species that have diverged evolutionarily suggests that it may have an important function (Bakker J and Baum MJ, 2000).

The pattern of GnRH release from the median eminence governs the various phases of mammalian reproduction. Work done with female monkeys first characterized GnRH secretion as "obligatorily intermittent" with periods of secretion followed by non-secretory periods (Knobil E, 1981). In most species studied, GnRH secretion is separated into basal/tonic secretion and surge-mode secretion. Tonic GnRH is secreted in distinct pulses with the amplitude and frequency changing in tune with changes in the reproductive cycle. A hypothalamic pulse generator initiates these pulses of GnRH. In contrast to basal GnRH secretion, GnRH surges occur prior to ovulation and are composed of high frequency pulses or continuous GnRH secretion. The preovulatory surge of GnRH and tonic GnRH pulses are the primary regulators of LH and FSH synthesis and secretion.

In ewes, portal blood vessel sampling has allowed direct measuring of GnRH release into the hypothalamus-hypophyseal blood system. Moenter and co-workers (1992), measured GnRH pulses and LH pulses and found that all of the significant LH pulses measured were immediately preceded by an increase in GnRH secretion. Furthermore, pulsatile administration of GnRH in the ewe leads to pulsatile LH release

(Clarke IJ and Cummins JT, 1982; Clarke IJ et al., 1984). As in sheep, the pulsatility of GnRH and LH secretion has been described in primates (Carmel PW et al., 1976), rodents (Levine JE and Ramirez VD, 1980), and humans (Santen RJ and Bardin CW, 1973). Episodic secretion of LH and GnRH has also been described in horses by cannulating the cavernous sinus and simultaneously measuring GnRH and LH (Irvine CHG and Alexander SL, 1994b). Studies using this technique have demonstrated a close correlation between GnRH, FSH, and LH secretion (Alexander SL and Irvine CHG, 1987); (Irvine CHG and Alexander SL, 1994b)). However, unlike sheep, primates, rodents, and humans, horses appears to have a less definite pattern of GnRH release. One study reported very irregular episodes of gonadotropic secretion in horses (Evans JW et al., 1979). Other studies described gonadotropin secretory events in mares as "episodes" rather than "pulses" due to their lack of rhythmical nature (Irvine CHG, 1995).

Another technique used to simultaneously measure GnRH and LH in horses is push-pull perfusion of the anterior pituitary gland. Grubaugh and co-workers (1987) studied GnRH release during various times of the year in pony mares using this technique (Grubaugh WR and Sharp DC, 1987). The secretory pattern of GnRH was monitored at ten-minute intervals and best described as "irregularly episodic but not regularly pulsatile." Further, not all GnRH secretory events preceded a rise in LH. The study concluded that the distinct secretory events occurred with no apparent regularity and were not circhoral. In addition, GnRH secretion in horses has been described as being episodic, with secretory events occurring two to three times per 12 h during diestrus and increasing in episode frequency during estrus, becoming continuous (Alexander SL and Irvine CHG, 1987). In a more recent study, evaluation of LH secretion revealed a pulsatile pattern of release in ewes and a non-pulsatile secretion of LH in pony mares (Porter MB et al., 1997b). In summation, the pattern of GnRH and LH secretion in horses does not appear to be strictly pulsatile making it different from that of other species.

The strict pulsatility of GnRH secretion, documented in species other than the horse, is critical for maintenance of responsive gonadotropes. In rodents, sheep, primates and humans, constant GnRH administration causes a cessation in LH secretion due to GnRH receptor desensitization and down-regulation (Catt KJ et al., 1983); (Marshall JC et al., 1983); (Nett TM et al., 1981); (Belchetz PE et al., 1978)). Receptor desensitization is described as a decline or loss of cellular response owing to continuous ligand exposure, which reflects dissociation between receptor and second messenger transducer (Dohlman HG et al., 1991). These changes reduce the cellular response even though receptors are present at the cell surface. In contrast, downregulation is a loss of cellular response due to reduction in the number of cell-surface receptors (Dohlman HG et al., 1991). Although desensitization and downregulation of the GnRH receptor have been described in other species, these phenomena have not been described in horse. Furthermore, in horses, continuous infusion of GnRH for 24 hours causes a steady rise in LH release (Garcia MC and Ginther OJ, 1975), plus the use of slow-release implants containing GnRH agonist elevated LH secretion for a period of 28 days (Turner JE and Irvine CHG, 1992). Similar data were described when pony mares were treated with GnRH for either 15 hours or 5 days (Porter MB et al., 1997b). In both experiments, mares responded to constant GnRH treatment with continued LH secretion. Moreover, during the mare's ovulatory surge, the rate of GnRH secretion increases approximately threefold over diestrus levels and is maintained at this elevated rate throughout estrus (Irvine CHG and Alexander SL, 1994b). Therefore, a distinct mechanism of GnRH secretion might exist in mares, which might correspond with variations in the gonadotropic signal transduction pathways in horses.

Regardless of the animal, the pattern of GnRH secretion is specific and necessary for an appropriate physiological response from the anterior pituitary. Similar to LH, FSH release is stimulated by GnRH. However, FSH secretion may also be highly regulated by gonadal steroids and specific gonadal peptides (Culler MD and Negro Vilar A, 1986). Furthermore, FSH secretion does not appear to be as strictly pulsatile as LH secretion but rather is slow and maintained relative to LH secretion. The disparity between secretory response of LH and FSH to GnRH may be due in part to FSH's longer half-life in the peripheral blood system.

GnRH Regulation

The substances that regulate GnRH release from its neurons may be organized into two categories. Category (A) includes neurotransmitters and neuromodulators, while category (B) includes steroid hormones and peptide hormones originating outside of the brain. Several of the primary substances in category (A) include: norepinephrine, dopamine, serotonin, endogenous opioid peptides, Gamma-aminobutyric acid (GABA), neuropeptide Y, and GnRH itself. Norepinephrine is a neurotransmitter in the catecholamine family that is involved in the generation of the preovulatory rise of LH via an alpha-adrenergic receptor (McCann SM et al., 1979). Blockade of alpha-adrenergic receptors resulted in suppression of pulsatile LH (Negro-Villar A et al., 1982); whereas infusion of norepinephrine through a push-pull cannula in rhesus monkeys stimulated GnRH release (Teresawa E et al., 1989). Furthermore, norepinephrine release has been characterized as pulsatile and synchronous with the pulsatile release of GnRH. Another member of the catecholamine family, dopamine has been shown to both stimulate and inhibit GnRH release. In vitro studies using hypothalamic tissue showed that infusion with dopamine stimulated GnRH release (Jarjeur L et al., 1986); however, previous studies using median eminence fragments infused with dopamine described an inhibition of GnRH release by dopamine. There are large concentrations of dopamine neurons in the tuberoinfundibular region that secrete dopamine suggesting a possible relationship with the neighboring GnRH neurons. In addition, there is extensive anatomical overlap in the distribution of GnRH neurons and serotonin-containing neurons in the hypothalamus (Jennes L et al., 1982). Serotonergic neurons may also play a stimulatory or inhibitory role in GnRH release. In sheep (Domanski E et al., 1975) and rats (Przkeop F et al., 1975), infusion of serotonin into the medial basal hypothalamus blocked the preovulatory surge of LH as well as ovulation. In contrast, one report demonstrated that a decrease in endogenous levels of serotonin tended to block spontaneous ovulation in rats (Lebhsetwar AP, 1972). More importantly, gonadal steroids appear to exert key influence on the actions of serotonin. In the presence of high estradiol (proestrus), serotonin appeared to stimulate GnRH release while in the absence of high estradiol (castrate or luteal) serotonin may inhibit release of GnRH (McEwen BS and Parsons B, 1982).

Beta-endorphin, an endogenous opioid peptide, inhibits GnRH release. The degree of opioid inhibition fluctuates with the gonadal steroid patterns of the estrous/menstrual cycle and has its greatest degree of inhibition during the luteal phase. When a beta-endorphin antagonist such as nalaxolone, was infused prior to ovulation, the preovulatory surge of LH was augmented (Sylvester PW et al., 1980), suggesting a strong inhibition of GnRH release by opioid peptides. Gamma-aminobutyric acid (GABA) is another substance that regulates GnRH release. The hypothalamus has a high concentration of GABA-producing neurons. The GABA-containing nerve terminals have synapses with GnRH containing neurons and may mediate their effect as a neurotransmitter. GABA appeared to inhibit the turnover rate of norepinephrine in the medial basal hypothalamus of rats (Adler B and Crowley W, 1986). In ovariectomized, estrogen-primed rats, GABA was measured by push-pull cannula and was shown to be diminished prior to the preovulatory LH-surge, whereas norepinephrine levels were shown to be elevated. Following ovulation, GABA levels were increased while both norepinephrine and GnRH levels were diminished. GABA neurons in the basal medial hypothalamus have been shown to contain estrogen receptors and it is proposed that some of GABA's negative effect on gonadotropin release is mediated by estradiol (Flugge G et al., 1986). Another regulatory substance that contains neurons closely associated with GnRH neurons is neuropeptide Y (NPY). It is proposed that NPY stimulates GnRH release. Infusion of rat medial basal hypothalamic and median eminence tissue with NPY stimulated GnRH release (Sabatino FD et al., 1989). Furthermore data suggest that NPY may be involved in mediating gonadal steroid feedback on GnRH neurons. Neuropeptide Y is partially regulated by gonadal steroids such that castration in rats results in a decline in NPY concentrations within the hypothalamus. This theory is supported by studies demonstrating that the estrogen-progesterone induced preovulatory surge can be blocked by injection of a NPY antibody into the third ventricle (Nehrenberg WB et al., 1989).

Unlike NPY, GnRH may inhibit its own secretion in an ultra-short feedback loop. Studies in rats demonstrated that GnRH agonist could inhibit endogenous GnRH release from the hypothalamus (Valenca MM et al., 1987). This study was done in castrated rats to remove any steroidal effects. This feedback mechanism in the rat may work through synaptic-like contacts between GnRH neurons as described by Valenca and Negro-Vilar (1984). Similar studies done in rams failed to identify an ultra-short feed back loop. Neither GnRH agonist nor GnRH antagonist had an effect on endogenous GnRH release in short-term castrated rams (Caraty A et al., 1990). The authors of this study postulated that the results of the rat studies, supporting an ultra-short feedback loop reflected pharmacological levels of GnRH agonist administered intra-ventricularly. Furthermore, a negative ultra-short feedback loop would not be physiologically advantageous during the preovulatory surge of LH, during which GnRH is secreted in large concentrations for several hours in ewes to several days in mares.

The second category of GnRH regulatory elements, in females, includes the gonadal steroids estrogen and progesterone. Removal of the ovaries causes a dramatic rise in GnRH release as well as LH and FSH release implicating the termination of inhibitory regulation by estrogen and progesterone on GnRH secretion. This has been observed in rats (Levine JE et al., 1982), ewes (Karsch FJ et al., 1977) and mares (Garcia MC and Ginther OJ, 1976). The steroids may be affecting the hypothalamus and/or the pituitary. In rats and rhesus monkeys, estrogen regulates gonadotropin release at the hypothalamus and the pituitary (Kalra PS and McCann SM, 1975); (Weick RF, 1978). Studies have shown that intra-ventricular injections of estrogen or implantation of estrogen implants in various hypothalamic nuclei suppress gonadotropin release (McCann SM, 1974); (Orias R et al., 1974). These data suggest a negative regulation of GnRH by estradiol, and correlate with the localization of estrogen-concentrating neurons in the arcuate region of the hypothalamus (Stump W, 1968). More recent work done in rats has shown that estrogen stimulates transcription of the GnRH gene in ovariectomized animals. Furthermore, an additive effect was detected when estrogen and progesterone were both given (Park OK, 1990).

It is unlikely that estradiol acts directly on GnRH secreting neurons because no estrogen receptors have been localized on these neurons. Rather, estrogen may be working indirectly by affecting levels of hypothalamic catecholamines and endogenous opioid peptides that in turn affect GnRH release. In rhesus monkeys it has been suggested that during proestrous, estrogen reaches a "threshold" concentration, which interrupts its negative feedback effect on GnRH release and facilitates the preovulatory surge of LH (Weick RF, 1978). Estrogen's action at the pituitary may be to increase the number and/or affinity of GnRH receptors plus stimulates the hypothalamus to release GnRH. Both sites of action work in synergism to provide the machinery for the preovulatory surge of LH. Similarly, studies in the ewe have shown that estradiol from the mature follicle has a positive feedback effect on GnRH release from the hypothalamus (Karsch FJ et al., 1983). However, during the period between luteolysis and the LH surge, estradiol negatively affects the pulsatile release of GnRH. Estradiol has also been shown to be a contributing factor in the LH surge at the level of the pituitary in ewes (Kaynard AH et al., 1988). Because of the difficulty in measuring GnRH in conscious mares, less information is known about the mare in comparison to rats, monkeys, and ewes. The majority of information known comes from studies measuring venous LH and FSH in response to exogenous gonadal steroids.

Sharp and co-workers (1991) measured the effect of estrogen and progesterone on LH and FSH secretion in ovariectomized pony mares during vernal transition. Exogenous estradiol was found to have a stimulatory effect on LH release and pituitary LH content (Sharp DC et al., 1991). This positive response was noted approximately one week after treatment began. Furthermore, when challenged with GnRH, there was no difference between control group and treatment group (E2), suggesting that estradiol may not have been acting at the pituitary to enhance it's responsiveness to GnRH but rather may have been acting at the level of the hypothalamus. Additionally, estradiol may have been working on LH synthesis directly as well. When progesterone was administered alone or in combination with estradiol, no effect was noted on LH release. However, upon GnRH challenge it was apparent that progesterone and progesterone+estradiol had a suppressive effect on circulating LH levels in the ovariectomized pony mare. Recent work by Greaves et al., (2000) demonstrated that estradiol treatment in ovariectomized mares stimulated an increase in LH secretion, beginning 4-5 days after initiating estrogen treatment, and continuing throughout estrogen treatment. Moreover, estrogen administration to pony mares following pituitary stalk sectioning did not increase LH concentrations suggesting that estradiol may not increase LH secretion directly via new synthesis in the pituitary gland (Porter MB et al., 1997a).

Along with the rise of estradiol during follicle maturation, the decline of progesterone during luteolysis plays an important role in the preovulatory LH surge. In ewes, progesterone levels are elevated in the periphery beginning several days after ovulation and persisting until day 13-14 of the cycle. During the luteal phase, progesterone exerts a negative effect on the interval between GnRH pulses. Following luteolysis, the GnRH pulse frequency increases and under the positive control of estradiol the preovulatory LH surge occurs (Karsch FJ et al., 1987). Similar to estradiol, progesterone receptors exist in the hypothalamus of the ewe and it has been suggested that progesterone may have its effect through the opioid peptide regulation of GnRH release. As mentioned previously, similar results have been documented in mares yet because of the difficulty in measuring GnRH directly, LH has been measured as an index of GnRH release. Garcia and Ginther (1978) demonstrated an inhibitory effect of progesterone on LH release in ovariectomized mares; however, the site of action was not elucidated. Catecholamine inputs as well as opioid peptides may be involved. In rats, progesterone rises prior to ovulation and appears to have a stimulatory effect on the LH-surge (Garcia MC and Ginther OJ, 1978). Kalra and co-workers (1974) observed a rise in progesterone levels before the rise in LH release (Kalra SP and Kalra PS, 1974). Furthermore, Ramirez (1987) administered progesterone to rats and observed a release of GnRH from the median eminence (Ramirez VD and Dluzen DE, 1987). Work done with epostan, a progesterone synthesis inhibitor, and RU-486, an anti-progesterone steroid, caused a pharmacological blockade of the LH surge (Rao IM and Mahesh VB, 1986); (DePaolo LV, 1988)). In all species mentioned, the temporal relationship between estrogen and progesterone is crucial for proper timing of the LH surge and ovulation.

GnRH Receptor

GnRH orchestrates reproduction by binding to its high affinity receptor (GnRH-R) expressed on the cell surface of gonadotropes in the anterior pituitary gland. Once bound to its receptor, the endocrine message sent from the hypothalamus is decoded resulting in the synthesis and release of LH and FSH from the gonadotropes. The gonadotropins (LH and FSH) in turn promote and sustain gonadal function. Normal reproductive cycles are dependent on timely fluctuations in the pattern of gonadotropin secretions, which are due in part to variations in gonadotropic response to GnRH secretion. These "variations" are mediated through regulation of GnRH receptor gene expression and receptor kinetics. Hence, the concentration and affinity of GnRH receptors present at the cell surface are the principle determinants of the physiologic response to GnRH secretion.

Gonadotropin-releasing hormone receptors are synthesized and expressed by gonadotropes located within the anterior pituitary gland. These are basophilic cells that represent a small percentage (8-15%) of the cell types located within the anterior pituitary gland. The other cell types include the somatotropes, mammotropes, thyrotropes, and corticotropes. The gonadotropes are distributed throughout the anterior pituitary gland nestled in a capillary plexus that demarcates the distal end of the portal vasculature. The portal vasculature originates within the median eminence of the hypothalamus and extends down the pituitary stalk to the anterior pituitary gland.

GnRH Receptor Structure

The amino acid sequence of the GnRH receptor has been determined for a variety of mammals including muridae (mice) (Tsutsumi M et al., 1992); (Reinhart J et al., 1992a); (MH Perrin et al., 1993), humans (Kakar SS et al., 1992); (L Chi et al., 1993), muridae (rats) (Eidne KA et al., 1992); (Kaiser UB et al., 1992) (MH Perrin et al., 1993), ovids (Brooks J et al., 1993) (Illing N et al., 1993), bovids (Kakar SS et al., 1993a) and porcids (Weesner GD and Matteri RL, 1994). The nucleotide and amino acid sequences of these clones are highly homologous. The mouse receptor is 97% homologous with the rat receptor, 89% homologous with the human receptor, and 87% homologous with the sheep receptor. The sheep and human receptor are 89% homologous with each other, partly due to one extra amino acid, a lysine residue in the second extracellular loop, present in these species compared to rat and mouse. Additionally, the nucleotide sequence for the GnRH receptor from several nonmammalian species has been recently isolated. These include the catfish, goldfish, clawed toad and chicken (Illing N et al., 1999); (Troskie BE et al., 1998a). These nonmammalian GnRH receptors have a high homology amongst themselves (> 70%) but are only 40% homologous with the GnRH receptor sequences from mammalian species. The protein structure of the mammalian GnRH receptor consists of 327 (rat and mouse)/ 328 (sheep, bovine, swine and human) amino acids and the calculated molecular weight is 37,684 daltons. Hydropathic analysis of the deduced amino acid sequences revealed seven hydrophobic regions. Thus, these receptors belong to a family of G-protein-coupled receptors characterized by an extracellular amino-terminal domain and seven hydrophobic segments connected by hydrophilic intracellular and extracellular loops. The N-terminus, extracellular loops and transmembrane domains are believed to be involved in ligand binding whereas the intracellular loops (second and third) and the C-terminus are important for G protein coupling and post-receptor modifications (Lin X et al., 1998b).

As a member of the G protein-coupled receptor family, the GnRH receptors share some of the same characteristics as other G protein-coupled receptors including a number of amino acid residues, which are highly conserved. These included prolines in the transmembrane domains 2, 4, 5, 6, and 7, which may play a role in ligand-induced signal propagation via a conformational change (kink formation) in the receptor (Davidson JS et al., 1994). Other conserved residues include Asn^{53} (TM1), Trp^{164} (TM4), Ser^{167} (TM4), Asn^{315} (TM7) and Tyr^{323} (TM7). Furthermore, as in many G protein-coupled receptors, there is a pair of cysteine residues that are believed to form a disulphide bridge between extracellular loop 1 and 2(Davidson JS et al., 1994). In addition to the cysteine residues, an arginine residue (Arg^{139}) is present in the third transmembrane domain of the GnRH receptor, as it is in most G protein-coupled receptors. Recent work by Ballesteros and co-workers (1998) demonstrated that not unlike other G protein-coupled receptors, Arg^{139} is required for efficient signal transduction (Lin X et al., 1998b). In their studies, mutation of this residue abolished GnRH-stimulated inositol phosphate generation.

Although the GnRH receptor is a member of the G protein-coupled receptor family, these receptors have low sequence homology with other G protein-coupled receptors. For example, unlike other members of the G-protein-coupled receptor family, mammalian GnRH-Rs do not contain an intracellular carboxy-terminus (Flanagan CA et al., 1997). In other receptors this region is important for agonist-induced phosphorylation, which may uncouple the receptor from its G protein and target the receptor for internalization (Ferguson SS et al., 1995; Lefkowitz RJ, 1998). Interestingly, nonmammalian GnRH receptors do contain a carboxy-terminus domain and the low homology between mammalian and nonmammalian species has been attributed to this variation. In nonmammalian species, as in other G-protein coupled receptors, the intracellular carboxy terminus plays an integral part in receptor desensitization and downregulation resulting from ligand-stimulated phosphorylation of Ser and Thr residues (Palczewski K and Benovic JL, 1991). Blomenrohr and co-workers (1999) demonstrated the importance of the carboxy terminus in the catfish GnRH receptor by studying the effect of progressive truncation of the carboxyl-terminal tail (Blomenrohr M et al., 1999). Their data indicated that as the carboxyl-terminal tail was increasingly truncated there was a decline in cell surface expression of the receptor. Furthermore, as the truncation involved the removal of GnRH-induced phosphorylation sites, the rate of receptor internalization diminished. Research done by Lin and co-workers demonstrated that the addition of the carboxy terminus from the catfish GnRH receptor to the rat GnRH receptor altered the pattern of receptor regulation from a biphasic down-regulation and recovery to a monophasic down-regulation (Lin X et al., 1998a). This alteration significantly enhanced the extent of receptor down-regulation in the rat GnRH receptor and it was reversed by removal of the added carboxy terminus. Lin and co-workers speculated that addition of the carboxy terminus to the rat GnRH receptor, which included 10 Ser and Thr residues, might have added additional phosphorylation sites, leading to increased receptor down-regulation. Alternatively, addition of the carboxy terminus may have caused a conformational change in the receptor leading to a decrease in receptor stability in the membrane and contribute to down-regulation. Even though mammalian GnRH receptors lack a cytoplasmic carboxy terminal domain, desensitization of the receptor has been described. One possible mechanism involves the possible phosphorylation sites that exist within the second and third intracellular loops. Lin and co-workers (1998) performed site-directed mutagenesis of potential phosphorylation sites for protein kinase C (Thr^{238}, Ser^{253}, and Thr^{264}) of the rat GnRH receptor (Lin X et al., 1998b). Results from this study suggested that these potential phosphorylation sites might play a role in the maintenance of structural integrity and expression of the GnRH receptor however phosphorylation of these residues does not appear to be a component of desensitization.

In addition to the lack of a cytoplasmic carboxy terminus, mammalian GnRH receptors have a modified amino acid motif at position 140 compared to other G protein-coupled receptors. At the cytosolic end of the third transmembrane helix and the beginning of the second cytoplasmic domain of the mammalian GnRH receptor, a Ser residue replaces the Tyr residue of the 'DRY' motif (Asp-Arg-Tyr). This motif is highly conserved among G protein-coupled receptors and has been implicated in the coupling of receptors with the G proteins (McArdle CA et al., 1995). Point mutation studies of the GnRH receptor have replaced the Ser residue with Tyr and no affect on coupling of the GnRH receptor to cytosolic G proteins was noted. However, the Tyr^{140} mutant receptor had an increased rate of receptor internalization and increased agonist binding affinity (Arora KK et al., 1995). The same study done by Arora and co-workers did identify conserved residues in this region of the GnRH receptor (Ile^{135}, Ile^{143} and Leu^{147}), which play a role in G protein coupling. Another unusual feature of the GnRH receptor is the substitution of aspartate and asparagine residues that are located in the second and seventh transmembrane domains in most other G protein-coupled receptors (Probst WC et al., 1992). In most G protein-coupled receptors, an aspartate residue in the second transmembrane domain is essential for agonist binding and G protein coupling. In the GnRH receptor, this residue is replaced by asparagine (Asn) ^{87}. Furthermore, in the seventh transmembrane domain a highly conserved asparagine is replaced by aspartate^{318} in the GnRH receptor. Mutation of the asparagine^{87} residue back to aspartate impairs ligand binding whereas mutation of the aspartate^{318} to asparagine significantly suppressed signal transduction of the GnRH receptor (Cook JV et al., 1993). If both residues were mutated back to their usual locations in G protein-coupled receptors, the receptors demonstrated minimal binding to ligand or signal transduction. These data suggested that Asn^{87} may be essential for ligand binding and although Asp^{318} may not play an important role in ligand binding, it is necessary for signal transduction. Additional studies have identified several other receptor residues (Asn^{102}, Lys^{121}, and Glu$^{301)}$ which are important for high-affinity binding to GnRH (Sealfon SC et al., 1997). These studies have shown that His^{2} of the decapeptide GnRH may interact with Lys^{121} near the top of transmembrane domain #3 whereas Arg^{8} of GnRH interacts with Glu^{301} in extracellular loop #3 and the c-terminal glycinamide of GnRH interacts with Asn^{102} at the apex of transmembrane domain #2. Additionally, recent work by Chauvin and co-workers (2000) demonstrated the importance of Trp^{279}, located within transmembrane domain #6, and Val^{299}, located in extracellular loop #3, for high-affinity binding to GnRH (Chauvin S et al., 2000). Identification of these residues as key components in binding between the GnRH receptor and GnRH provided sufficient information for Chauvin and co-workers to predict a three-dimensional model of the GnRH receptor binding with its ligand, GnRH. In this model, it is assumed that the GnRH receptor has a conformation not unlike rhodopsin in which the transmembrane bundle is oriented such that Asn^{87} and Asp^{318} are in a position to interact with each other and a disulfide bond may exist between Cys^{114} and Cys^{195}. These Cys residues are highly conserved among the majority of G protein-coupled receptors. This conformation also allows for the first residue of GnRH, PyroGlu^{1}, to interact with the base of the central receptor cleft near transmembrane domain #5. Furthermore, His^{2} of GnRH and Lys^{121} of the receptor fall into alignment and in turn, Arg^{8} and Trp^{3} of GnRH interact with Glu^{301} and Trp^{279} of the receptor, respectively. Site-directed mutagenesis of any or all of these residues results in a significant decline in ligand binding which does not appear to be caused by a decline in receptor number but rather a decrease in binding to cell surface receptors. Yet another residue of the GnRH receptor, which is a key component of ligand binding, appears to be the highly conserved Cys residue at the extracellular edge of transmembrane domain #3. This Cys residue probably forms a disulfide bond with one of the Cys residues in transmembrane domain #2 thus stabilizing the GnRH receptor-ligand complex. Work done by Keinan and Hazum (1995) demonstrated that disruption of disulfide bridge formation with a reducing agent significantly reduced the receptor's affinity for GnRH (Keinan D and Hazum E, 1985).

Another example of the mammalian GnRH receptor's uniqueness with respect to other members in the family of G protein-coupled receptors was elucidated in work done by Myburgh and co-workers (1998). In that study, scientist mutated six sites in the human GnRH receptor, corresponding to amino acid residues which, when mutated in other G protein-coupled receptors lead to constitutive activity. None of the mutants caused constitutive activity in the human GnRH receptor suggesting a different mechanism of activation in GnRH receptors compared to other G protein-coupled receptors (Myburgh DB et al., 1998b).

Although the mammalian GnRH receptor differs in some respects from other G protein-coupled receptors, there exist similarities between these receptors and other G protein-coupled receptors. For example, mammalian GnRH receptors have several potential glycosylation sites, including Asn ^{4}, Asn ^{18} and Asn ^{102}. Of these three potential glycosylation sites, only two are highly conserved amongst mammalian GnRH receptors, Asn ^{18} and Asn ^{102} (Davidson JS et al., 1994). Work by Davidson et al., (1994) demonstrated that mutation of these Asn residues to Gln resulted in a minor change in affinity but a significant decline in total binding was noted. They concluded that glycosylation of the GnRH receptor was not directly involved in ligand binding but rather was important for providing an adequate concentration of receptors at the cell surface. This data supported early work done by Shavartz and Hazum (1985), which indicated that inhibition of *N*-glycosylation at these residues caused a decline in the density of rat GnRH receptors but not a change in affinity of the receptor for ligand. Therefore, inhibition of post-translational glycosylation appears to impair the localization of GnRH receptors to the plasma membrane.

Another feature of the GnRH receptor, which is typical of other members of the G protein-coupled receptor family, is the presence of the hydrophobic amino acid Leu^{237} at the N-terminal region of intracellular loop #3. This amino acid is located a constant distance from two highly conserved amino acids, Pro and Try, in transmembrane domain #5. Site-directed mutagenesis studies revealed that, like other G protein-coupled receptors, Leu^{237} is critical for G protein coupling of the GnRH receptor, subsequent initiation of signal transduction, and receptor internalization (Chung H-Y et al., 1999).

GnRH Receptor Tissue Expression

Cloning of the GnRH receptor has provided valuable information regarding the nucleotide structure and potential mechanism of action. Additionally, it has given scientists the means where by expression of GnRH receptor can be detected and quantified. Initially, this was limited to the anterior pituitary gland since it is thought to be the primary target for hypothalamic GnRH. Among the anterior pituitary cells, GnRH binding sites are located exclusively in gonadotropes. These include gonadotropes expressing both LH and FSH and gonadotropes expressing only LH or FSH (Naor Z and

Childs GV, 1986). However, Northern blot analyses and reverse transcriptase polymerase chain reaction (RT-PCR) have identified mRNAs encoding for the GnRH receptor in extrapituitary tissue. Kakar and Jennes (1995) used RT-PCR to screen various human reproductive and non-reproductive tissues (Jennes L et al., 1985). PCR products of expected size were identified in human liver, heart, skeletal muscle, kidney, placenta, and pituitary. Southern blot analysis was used to confirm the authenticity of the PCR products. The PCR product synthesized from the heart RNA was cloned and sequenced, revealing sequence identical to the GnRH receptor of the pituitary gland. In addition to these tissues, scientists have reported the presence of GnRH mRNA in human ovaries (Peng C et al., 1994), breast (Dong KW et al., 1993), testes (Dong KW et al., 1993), as well as in several human tumors and tumor cell lines, including ovarian and breast tumors (Kakar SS et al., 1994b). Interestingly, GnRH agonists and antagonist have been shown to inhibit cell growth and [^{3}H] thymidine incorporation in tumor cells (in vitro) (Eidne KA et al., 1987). Thus, two mechanisms may be involved with the tumor suppression effect of GnRH treatment in humans. The first being, that GnRH antitumoral treatment down regulates the hypothalamic-pituitary-gonadal axis and therefore suppresses steroid hormone production. Secondly, the presence of GnRH receptors directly on the tumors may allow GnRH treatment to have a direct effect on the tumor itself by suppressing cell growth. More recent work done by Kottler and co-workers (1999) has reported finding GnRH receptor mRNA in human testis, granulose cell tumors, and breast tissue (cancerous and non-cancerous). No mRNA for the GnRH receptor was detected in uteri, intestine or leucocytes (Kottler ML et al., 1999). Furthermore, Kottler and co-workers identified 3 different mRNA species for the human GnRH receptor, which appeared to be a result of variation in gene splicing. Depending on the tissue, one or a combination of the 3 mRNA species was identified using RT-PCR and Southern blotting techniques. In other studies, Northern blot analysis of human tissue identified a 4.7 to 5.0 kb primary transcript and two faint bands of 2.5 and 1.5 kb were also identified (Chi L et al., 1993). In the mouse, 3.5 and 1.6 kb transcripts were identified whereas in the rat a 4.5 kb transcript was predominant (Reinhart J et al., 1992b). In sheep, Northern blot analysis hybridized with transcripts ranging in size from 1.3 kb to 6 kb (Brooks J et al., 1993). These data suggest that GnRH receptor expression may be regulated in a tissue- and species-specific manner.

Kang and co-workers (2000) strengthened this theory by quantifying the expression of GnRH receptor mRNA in pituitary cells versus ovarian carcinoma cells (Kang SK et al., 2000). A nine-fold increase in expression level was noted in pituitary cells compared to ovarian cells. Further analysis revealed that the difference in gene expression could be attributed, in part, to binding of different cell-specific regulatory factors (nuclear proteins) to various promoter elements in the 5′-flanking region of the gene. A lower level of receptor expression has also been demonstrated in the rat ovary and testes versus the pituitary gland (Kakar SS et al., 1994a). Likewise, expression of the human GnRH receptor appears to be 200-fold greater in the pituitary compared to granulosa-luteal cells (Minaretzis D et al., 1995).

Similar studies were performed in bovine tissue, however mRNA for the GnRH receptor was detected only in the pituitary gland and not in the hypothalamus, testis, pancreas, spleen, hippocampus, liver, adrenal or kidney (Kakar SS et al., 1993b). Concerned that Northern blot analysis was not sensitive enough to detect the mRNA in these extrapituitary tissue, Kakar and co-workers performed RT-PCR on the above-mentioned bovine tissue. Nonetheless, RT-PCR of the bovine pituitary tissue produced only a band consistent with the GnRH receptor. In contrast, GnRH receptor mRNA, as detected by Northern blot analysis, was identified in rat pituitary gland, hippocampus, hypothalamus, ovary, and Leydig cells (not sertoli cells) (Reinhart J et al., 1992b). In the mouse, immunohistochemistry of various tissues revealed antibody binding in the anterior pituitary gland and the endometrium, whereas there was no binding in ovary, brain, liver, kidneys, heart, lungs, or spleen. Earlier studies using autoradiography localized GnRH receptors in various parts of the brain including the hippocampus, lateral septal nucleus, anterior cingulated cortex, subiculum, and hypothalamus (Millan M et al., 1985). Admittedly, immunohistochemistry and autoradiography are not as sensitive as RT-PCR. However, the need to use such a sensitive procedure to identify the presence of GnRH receptor mRNA in extrapituitary tissues may bring in to question the physiologic relevance of these findings.

GnRH Receptor Gene

The human (Fan NC et al., 1994) and mouse (Kaiser UB et al., 1992) GnRH receptor genes have been localized to chromosomes 4 and 5, respectively. Cloning and analysis of the gene that encodes for the human GnRH receptor revealed that structurally, the receptor gene is made up of three exons and two introns, spanning over 20 kb in size (Fan NC et al., 1995) (Kakar SS, 1997). Exon I contains the 5′ untranslated region and a portion of the open reading frame encompassing transmembrane domains I, II, III and a portion of IV (nucleotide +1 to +522). Exon II encodes the remainder of transmembrane domain IV and all of V (nucleotide +523 to +742), while exon III encodes for the remainder of the open reading frame as well as the 3′ untranslated region (nucleotide +743 to +987). The structures of the mouse (Zhou W and Sealfon SC, 1994), rat (Reinhart J and Catt KJ, 1995), and sheep (Campion CE et al., 1996) GnRH receptor gene have also been characterized and all contain three exons and two introns. Examination of the 5′ end of human GnRH receptor gene identified five consensus TATA boxes (TATAAA) located in close proximity to each other and several CAAT boxes were found scattered among the TATA boxes (Fan NC et al., 1995). The presence of several TATA boxes in the 5′ region of the gene indicates multiple transcription initiation sites. Fan and co-workers (1995) identified 5 transcription initiation sites (+1, -101, -124, -669, and –690), which were in close proximity to TATA boxes. Similar to the human GnRH receptor gene, the ovine gene appears to have multiple transcription sites approximately 800 bp upstream of the translation start codon (Campion CE et al., 1996). Interestingly, comparison of the 5′ flanking region of the human and sheep GnRH receptor genes revealed high (70%) sequence homology. In contrast, there is only 46% homology between the human GnRH receptor gene and the mouse GnRH receptor gene. This is due in part to the presence of the transcription start site much closer to the translation start site (62 bp upstream) (Albarracin CT et al., 1994). Analysis of the mouse GnRH receptor gene revealed that one of the two transcription start sites appears to be TATA-less whereas the other does not. However both transcription start sites contribute to full transcriptional activity of the mouse GnRH receptor gene (Clay CM et al., 1995). The primary transcription start site in the rat GnRH receptor gene has been localized approximately 104 bp upstream of the start codon and appears to be associated with a TATA box (Reinhart J and Catt KJ, 1995). The presence of several transcription initiation sites suggests that the human and mouse GnRH receptor gene may be highly regulated and may express variable size transcripts. These data suggest the possibility of species-specific or tissue-specific transcription initiation sites, giving rise to different transcript sizes depending on species and/or tissue. Furthermore, Northern blot analyses of human (Kakar SS, 1997), bovine (Kakar SS et al., 1993b), rat (Kakar SS et al., 1994a), sheep (Illing N et al., 1993) and mouse (Tsutsumi M et al., 1992) RNA have revealed several hybridizing mRNAs with sizes ranging from 1.5 kb to 5.0 kb, which appear to contain full-length coding sequences. These data are consistent with the idea of multiple transcript expression.

Additionally, analysis of the GnRH receptor's promoter region have identified several binding sites for regulatory elements in the GnRH receptor gene. These include a number of consensus *cis*-acting regulatory sequences in the 5′ end of the GnRH receptor gene (Fan NC et al., 1995). One example includes the identification of two PAE-3 binding sites upstream from the TATA boxes, which are thought to act as phorbol ester binding sites (Xin JH et al., 1992). In addition to the PAE binding sites, Activator protein 1 (AP-1) and Activator protein 2 (AP-2) binding sites have been localized in the human (Imagawa M et al., 1987), mouse (Albarracin CT et al., 1994) and rat (Reinhart J and Catt KJ, 1995) GnRH receptor. AP-1 is a heterodimer made up of fos, jun, and/or activating transcription factor (ATF), which is stimulated by Protein Kinase C (PKC). Several investigators have studied the gonadotrope-derived αT3-1 cell line, which is murine in origin and expresses GnRH receptors and the gonadotrope alpha subunit (Windle JJ et al., 1990). These cells were derived from pituitary tumors in transgenic mice, which had been treated with a fusion gene containing a portion of the human glycoprotein hormone α-subunit gene linked to the protein-coding sequences of the simian virus-40 T antigen oncogene. Duval and co-workers (1997) identified the AP-1 binding site as one of 3 in a tripartite basal enhancer of the murine GnRH receptor gene (Duval DL et al., 1997). Using αT3-1 cells in deletion and mutational studies in the proximal promoter; the tripartite basal enhancer was described as containing binding sites for AP-1, steroidogenic factor-1 (SF-1), and a novel element termed GnRH receptor activating sequence (GRAS). The AP-1 binding site was located at –336 to –330 bp from the translation start site, the SF-1 binding site was located at –393 bp and the GRAS element was identified between –393 and –386 bp. Mutation of each binding site alone resulted in a 60% loss of promoter activity, whereas combinatorial mutations of any two binding sites decreased promoter activity by 80%. If all binding sites were mutated at once, the promoter was rendered useless (Duval DL et al., 1997). More recently, Norwitz and co-workers (1999) used deletion and mutational studies in the murine gonadotrope-derived αT3-1 cell line to determine which regions of the GnRH promoter region are responsive to GnRH (Norwitz ER et al., 1999). The transfection of αT3-1 cells with serial deletion mutants of the 5′- flanking region (-1164 to +62) of the murine GnRH gene identified elements within the region –300 to -232 that are necessary for GnRH responsiveness. Closer examination identified the region -276 to -269 as containing the consensus sequence for the AP-1 binding site and the region –292 to -285 as containing the sequence for a novel element. The AP-1 binding site was labeled SURG-2 and the novel element, SURG-1. To determine if these regions of the murine GnRH receptor promoter region were necessary for conferring GnRH responsiveness to the mouse GnRH receptor gene, point mutation studies of the SURG-1 and SURG-2 regions were performed. Mutation of SURG-2 (AP-1 binding site) completely abolished the GnRH agonist stimulated response. However, mutation of SURG-1 only diminished the response. Furthermore, these two elements, either combined or alone, are sufficient to elicit activity of a heterologous minimal promoter, GH_{50}, in αT3-1 cells. These data implicate the AP-1 binding site of the murine GnRH receptor promoter as critical to GnRH responsiveness whereas the SURG-1 element may be necessary for a full GnRH response. Treatment of αT3-1 cells transfected with –308 to -264 (SURG-1 & SURG-2 regions) of the murine GnRH receptor gene with Phorbol 12-myristate 13-acetate (PMA) resulted in similar luciferase activity as cells treated with a GnRH agonist. There was an additive effect of cells treated with PMA and a GnRH agonist. Furthermore, treatment with an inhibitor of PKC isoforms (GF-109203X) resulted in no luciferase activity. These data suggest that both PMA and GnRH-agonist stimulation of the GnRH receptor gene is mediated through the activation of PKC. However, the additive effect of treating cells with both PMA and GnRH agonist indicates that these agents act through different PKC isoforms or that they act synergistically (Norwitz ER et al., 1999).

Another consensus sequence is the binding site for Pit-1, an anterior pituitary specific transcription factor. This transcription factor has been implicated in the regulation of certain pituitary hormones, including growth hormone (Bodner M et al., 1988) and prolactin (Ingraham HA et al., 1990). A Pit-1 binding site has been identified in the human (Fan NC et al., 1995) and rat (Reinhart J and Catt KJ, 1995) GnRH receptor gene, supporting the theory that tissue specific (anterior pituitary gland) regulation of GnRH receptor transcription may exist. This theory is further strengthened by transfection studies that demonstrated the GnRH receptor promoter activity is significantly higher in pituitary cells than non-pituitary derived cells (Albarracin CT et al., 1994; Clay CM et al., 1995). Recent work has identified the existence of a cyclic AMP (camp) response element (CRE) and a glucocorticoid/progesterone response element (GRE/PRE) (Kakar SS, 1997). At the 3′ end of the GnRH receptor gene five "AATAAA" polyadenylated signals have been identified, located throughout a region of 800 nucleotides (Fan NC et al., 1995).

As mentioned previously, the homology of the 5′ flanking region of the GnRH receptor tends to vary amongst species (40-70%). For example, the human GnRH receptor gene appears to be more complex than that of other species whereas the ovine GnRH receptor gene promoter region appears to lack a consensus AP-1 binding site (Campion CE et al., 1996). Interestingly, the SURG-1 and SURG-2 consensus sequences tend to be conserved in the mouse, rat, and human. These findings suggest that different mechanisms may be involved in the GnRH-mediated activation of the GnRH receptor gene in different species.

GnRH Receptor Gene Regulation

In addition to the consensus *cis*-acting regulatory sequences in the 5′ end of the GnRH receptor, the GnRH receptor gene is influenced by a variety of hormones including, steroids, gonadal peptides, second messenger activators and GnRH itself. Estradiol appears to have a species-specific regulation of GnRH receptor expression. In αT3-1 cells, estradiol caused a reduction in GnRH receptor number however in rat pituitary cells both an increase and decrease in GnRH receptor number were documented (Emons G et al., 1988; McArdle CA et al., 1992). *In vivo* experiments in rats, which were ovariectomized and then treated with estradiol 21 days later, reported a significant decline in GnRH receptor mRNA by 7 days (Kaiser UB et al., 1993). In contrast, ovine pituitary cells treated with estradiol responded with an increase in GnRH receptors, which was maintained for over 48 hours (Laws SC et al., 1990). Gregg and co-workers (1990) obtained similar data by treating primary cultures of ovine pituitary cells with estradiol, which resulted in dose-and time-dependent increase in number of GnRH receptors (Laws SC et al., 1990). Furthermore, actinomycin D and cyclohexamide treatment abolished the estrogen-induced rise in GnRH receptor number suggesting that both transcription and translation are involved in estrogen's upregulation of the GnRH receptor. Hence, estradiol regulatory effect on ovine GnRH receptor expression may be genomic. *In vivo* data from orchidectomized sheep reported a significant rise in tissue levels of GnRH receptor mRNA following infusion with estradiol, which was dose-dependent (Gregg DW et al., 1990); (Sakurai H et al., 1997). Furthermore, withdrawal of estrogen treatment resulted in a rapid decline in concentrations of GnRH receptor mRNA (Adams BM et al., 1996). The withdrawal of estrogen is also associated with a reduction in the concentration of GnRH receptors. The decline in receptor number suggests that estrogen withdrawal may destabilize GnRH receptor mRNA and/or may reduce the rate of transcription (Sakurai H et al., 1997).

The effect of progesterone appears to be inhibitory on the expression of GnRH receptors. Data obtained from *in vivo* rat studies revealed a significant increase in GnRH receptor mRNA due to estrogen treatment of ovariectomized rats, however progesterone treatment decreased steady state levels of GnRH receptor mRNA (Adams BM et al., 1996). Additionally, Sealfon and co-workers (1990) reported an inhibitory effect of progesterone on GnRH receptor concentration when ovine pituitary cells in culture were treated with progesterone (Bauer-Dentoin AC et al., 1995). More recent data from studies evaluating the effect of progesterone treatment in orchidectomized sheep, indicated that pituitary tissue concentrations of GnRH receptor number and mRNA were significantly reduced in wethers implanted with progesterone (Sakurai H et al., 1997). This progesterone-induced decline in GnRH receptor activity was not reversed within 24 hours of progesterone withdrawal. The inhibitory effect of progesterone on pituitary GnRH receptor mRNA was corroborated by Wu and co-workers (1994). Treatment of ovine pituitary cell cultures with progesterone resulted in a 50% reduction in levels of GnRH receptor mRNA (Wu JC et al., 1994). In contrast, Greg and co-workers reported that treatment of ovine pituitary cells with progesterone plus estradiol resulted in an additive increase in GnRH receptor number. However, progesterone alone did not cause an increase in receptor number suggesting that progesterone may potentiate estradiol's ability to increase receptor number. Similar work done in ewes demonstrated that treatment of OVX ewes with estradiol in the absence of progesterone increased amounts of GnRH receptor mRNA threefold however ewes treated with progesterone and estradiol responded with an only a 2-fold increase in mRNA levels (Turzillo AM et al., 1998a). Additionally, treatment of ewes during the luteal phase with estradiol did not increase steady-state amounts of GnRH receptor. To better understand the interactions of progesterone and estradiol on levels of mRNA for GnRH receptors in sheep, Kirkpatrick and co-workers (1998) devised a study that investigated the effect of progesterone withdrawal in the presence of estradiol. This design was meant to replicate the natural events occurring following luteolysis (progesterone withdrawal) during which estradiol levels are rising. Data from this study indicated that removal of progesterone in the presence of estradiol resulted in increased amounts of mRNA for GnRH receptors, but did not affect the number of GnRH receptors (Kirkpatrick BL et al., 1998). The authors summarized that the increase in GnRH receptor concentration, prior to the preovulatory surge of GnRH in sheep, might be due in part to the declining progesterone concentrations and increasing estradiol concentrations.

The data from *in vitro* experiments and *in vivo* experiments in ovariectomized or castrated animals are supported by hormonal changes taking place throughout the estrous cycle. During the periovulatory period, increasing estradiol secretion from the preovulatory follicle results in increased concentration of pituitary GnRH receptor (Adams BM et al., 1996). In ewes, tissue concentrations of both GnRH receptor and GnRH receptor mRNA increase with increasing endogenous estradiol (Sakurai H et al., 1997). The concentration of GnRH receptor mRNA in sheep is significantly higher during the estrus phase as compared to diestrus. In one study, concentrations of GnRH receptor mRNA increased significantly within 12 hours after the induction of luteolysis (Turzillo AM et al., 1994). This increase in GnRH receptor mRNA was associated with declining progesterone levels and occurred before measurable changes in circulating estradiol concentrations. Turzillo and Nett (1999) suggested that declining levels of progesterone, at luteolysis, initiate the molecular events responsible for increased concentrations of GnRH receptor mRNA. Subsequently, as the pre-ovulatory follicle matures, rising levels of estradiol may function to maintain elevated GnRH receptor mRNA and receptor number (Turzillo AM and Nett TM, 1999). The increasing level of GnRH receptor mRNA is maximal just before ovulation and drops significantly at the time of the GnRH surge (Cowley MA et al., 1998). Similar data have been reported during the rat estrous cycle (Clayton RN et al., 1980). These physiologic data support the theory that estradiol up regulates the expression of GnRH receptors during the periovulatory period whereas progesterone may inhibit the expression of the GnRH receptor during the luteal phase. The pre-ovulatory upregulation of pituitary GnRH receptor heightens the sensitivity of the anterior pituitary gland to GnRH in preparation for the pre-ovulatory GnRH surge and ovulation.

The ovarian steroids, progesterone and estradiol, are not the only ovarian hormones which influence the expression of pituitary GnRH receptors. Activin A has been shown to increase GnRH receptor mRNA in αT3-1 cells in a time- and dose-dependent manner (Fernandez-Vasquez G et al., 1996). Furthermore, pretreatment of αT3-1 cells with Activin A enhanced the GnRH-induced activation of the gonadotropin α-subunit promoter, presumably by increasing the pituitary's responsiveness to GnRH. Duval and co-workers (1999) identified a region of the murine GnRH receptor gene promoter which confers activin responsiveness (Duval DL et al., 1999). Their data indicated that the 600 bp of 5′-flanking sequences from the mouse GnRH receptor gene (αT3-1 cells) was adequate to confer activin responsiveness. As mentioned previously, included within the 600 bp promo there are three *cis*-acting elements critical for expression in αT3-1 cells. These include SF-1, AP-1 and GRAS binding sites. A construct consisting of 3 copies of GRAS located upstream of a heterologous minimal promoter was responsive to activin stimulation, implicating GRAS as an important factor in activin regulation of GnRH receptor gene expression. Moreover, mutation of the GRAS region resulted in an inability of the promoter to respond to follistatin, an activin-binding protein. This was an important observation since increasing concentrations of follistatin resulted in a dose-dependent decrease in promoter activity.

In contrast to results obtained with the mouse GnRH receptor, research done on cultured ovine pituitary cells reported that recombinant Activin treatment did not affect GnRH receptor number, however, treatment of ovine cells with inhibin resulted in an increase in GnRH receptor number. This affect of inhibin was enhanced if ovine pituitary cells were simultaneously treated with estradiol (Gregg DW et al., 1991). The authors of this study concluded that, in conjunction with estradiol, inhibin might be preparing the pituitary for the preovulatory surge of LH by increasing GnRH receptor number hence enhancing the sensitivity of the pituitary to GnRH.

One of the most important regulators of GnRH receptor expression is GnRH itself. Homologous regulation of the GnRH receptor by GnRH has been reported to occur *in vivo* (Clayton RN and Catt KJ, 1981) in rats and *in vitro* in cultured rat pituitary cells (Loumaye E and Catt KJ, 1982). More recent work by Cheon and co-workers (1999) demonstrated an increase in GnRH receptor mRNA following treatment of cultured rat pituitary cells with low, continuous doses of GnRH (Cheon M et al., 1999). Furthermore, these studies showed that when pituitary cells were treated for 6 hours with either a continuous mode of GnRH or hourly pulses of GnRH, both treatments resulted in a significant rise in GnRH receptor mRNA levels. In contrast, if pituitary cells were treated for 25 hours with continuous GnRH, no change in GnRH receptor mRNA was noted. These findings support earlier work done in cultured rat pituitary cells which reported that hourly pulses of GnRH increased GnRH receptor mRNA whereas continuous GnRH treatment had no effect of steady-state levels of GnRH receptor mRNA (Kaiser UB et al., 1993).

Upregulation of the GnRH receptor has also been documented in murine αT3-1 cells. Brief exposure (20 min.) of αT3-1 cells to GnRH resulted in a 50% increase in the density of GnRH receptors with no apparent change in GnRH receptor mRNA (Tsutsumi M et al., 1993). Treatment of αT3-1 cells with GnRH for 24 hours resulted in a 75% decline in GnRH receptor number, however no apparent change in concentration of GnRH receptor mRNA was detected (Tsutsumi M et al., 1995). Quantitative assessment of the GnRH receptor mRNA indicated that although there was no change in mRNA concentrations, the decline in receptor number was associated with a decline in translation of the GnRH receptor. White and co-workers (1999) investigated the molecular mechanism underlying GnRH regulation of GnRH receptor gene expression in αT3-1 cells. Mutation of the AP-1 binding site within the GnRH receptor's promoter region abolished GnRH responsiveness, suggesting that the GnRH upregulation of its own receptor might be mediated through the activation of the AP-1 binding site (White BR et al., 1999).

In addition to the presence of key 5′- flanking consensus sequences, such as AP-1 binding site, the mode (continuous versus pulsatile) of GnRH secretion or administration is paramount to the regulation of GnRH receptor and gonadotrope response in most species. In cows, continuous infusion of GnRH caused a decline in concentrations of GnRH receptor number and mRNA (Vizcarra JA et al., 1997). Similarly, high-amplitude GnRH stimulation of orchidectomized sheep resulted in a significant decline in tissue concentrations of GnRH receptor mRNA (Adams BM et al., 1996). Moreover, treatment of ovariectomized ewes with continuous GnRH resulted in decreased levels of GnRH receptor mRNA and concentration of receptors (Turzillo AM et al., 1998b). Interestingly, estradiol treatment was able to override the negative effect of constant GnRH by stimulating an increase in GnRH receptor gene expression and GnRH receptor density. These data agree with studies that assessed continuous vs episodic GnRH exposure. Continuous GnRH exposure and high agonist concentration results in downregulation or a decline in GnRH receptor density (McArdle CA et al., 1987) whereas episodic GnRH exposure and low agonist concentrations tended to increase the concentration of GnRH receptor number (Katt JA et al., 1985).

Besides the importance of pulsatile GnRH exposure for maintenance of GnRH receptor expression, second messenger effectors, such as calcium, may also play a role in regulation of the GnRH receptor gene. Haisenleder and co-workers demonstrated that pulsatile calcium influx in cultured rat pituitary cells resulted in a rise in mRNA encoding the GnRH receptor. This rise was not different from that noted when the cells were treated with pulsatile GnRH (Haisenleder DJ et al., 1997). In addition to calcium, the effects of the second messenger activators, PMA and forskolin have been evaluated (Alarid ET and Mellon PL, 1995). Exposure of αT3-1 cells to PMA, a phorbol ester that activates protein kinase C (PKC), had no effect on GnRH receptor mRNA. However forskolin exposure, which activates adenylyl cyclase, resulted in a decrease in GnRH mRNA. A more recent study evaluated the role of mitogen-activated protein kinase (MAPK) activation on GnRH receptor gene expression in cultured rat pituitary cells (Haisenleder DJ et al., 1998). Exposure of rat cells to a MAPK inhibitor resulted in a suppression of GnRH receptor mRNA response to pulsatile GnRH treatment. These studies suggest that the GnRH receptor gene is highly regulated at various points in the transduction of the signal from GnRH to the gonadotrope.

GnRH Receptor Ligand Interactions

The binding of GnRH to its high affinity receptor results in receptor dimerization and aggregation into coated pits throughout the plasma membrane of pituitary gonadotropes (Conn, 1982). Data from Janovick and Conn (1996) reinforced the idea of receptor dimerization by describing receptor microaggregation immediately following GnRH binding to its receptor (Janovick JA and Conn PM, 1996). The recent development of the GnRH receptor gene constructs fused with the green fluorescent protein (GFP) reporter molecule, from jellyfish *Aequorea Victoria*, has allowed visualization of unoccupied and occupied GnRHs receptor in living cells (Prasher DC et al., 1992). In one study, stimulation of rat GFP-bound pituitary cells with a GnRH agonist resulted in an exchange of fluorescence between the cytoplasm and the plasma membrane and fluorescent spots could be seen fusing with the membrane and internalizing (Xinwei L et al., 1998). In another study, the lateral mobility of GnRH receptors within the plasma membrane was investigated. In the unbound state most of the GnRH receptors were laterally mobile. However, binding of GnRH to the receptor significantly reduced the rate of lateral diffusion contributing to aggregation of the receptor (Nelson S et al., 1999).

Association with coated vesicles and internalization follows dimerization of receptor-ligand complexes via endocytosis. Quantitative autoradiographic studies of cultured rat pituitary cells demonstrated that, after binding with receptors on the plasma membrane, GnRH is rapidly internalized, accumulating in secretory granules, and localizing over the nuclear membrane and later, in the nucleus (Morel G et al., 1987). Additional studies using the GFP conjugates of the GnRH receptor demonstrated that the receptor-ligand complex is internalized within a vesicle and subsequently the agonist (GnRH) separates from receptor as the vesicle approaches the perinuclear space (Cornea A et al., 1999). In this study, GH_3 cells tagged with GFP were treated with Texas Red labeled GnRH agonist. This allowed the simultaneous monitoring of receptor and ligand fate following endocytosis. Cornea and co-workers reported that the internalization process continued for 2-3 hours, at which time no visible agonist (red colored) remained bound to the cell surface. The rate of internalization was calculated (0.5%/minute), representing the fraction of total receptor and agonist associated with one cell, which is internalized each minute, in one cell. Once all visible agonist was internalized, the concentration of free receptor on the plasma membrane (green colored) began to increase, presumably through newly synthesized receptor and/or from recycling of previously internalized receptor. Preincubation of cells with the protein synthesis inhibitor, cyclohexamide resulted in no direct effect on the rate of receptor recycling. These data suggest that the primary source of cell-surface receptor replenishment is that of internalized receptors which have been free of agonist.

Internalization of the receptor-ligand complex is a common feature of G protein-coupled receptors. The binding of β-arrestins to G protein-coupled receptors has been shown to be a key component in regulating the rate and specificity of receptor internalization (Ferguson SS et al., 1996). In the beta2-adrenergic receptor, arrestin promotes the internalization of the receptor by binding with clathrin, the major structural protein in coated pits (Goodman OB Jr et al., 1996). β-arrestins are proteins that bind phosphorylated heterotrimeric GTP-binding protein-coupled receptors and contribute to desensitization of G protein coupled receptors by uncoupling the signal transduction pathway (Ferguson SS et al., 1996). G protein-coupled receptor kinases (GRKs) are responsible for phosphorylating the intracellular regions of seven-transmembrane receptors, permitting β-arrestins to bind. The role of β-arrestin in GnRH receptor internalization was examined using HEK 293 cells. These cells expressed the rat GnRH receptor epitope-tagged with a fluorescent label. Using confocal microscopy the surface-bound and subcellular location of GnRH receptors was monitored. Agonist stimulation of these cells resulted in reversible receptor redistribution from the plasma membrane into the cytoplasm, beginning after 20 minutes and nearly complete within 2 hours post-agonist stimulation (Vrecl M et al., 1998). Once the agonist was removed, receptor recycling was noted within 20 minutes and by 60 minutes nearly all internalized receptor was at the cell surface. However, co-expression of the GnRH receptor with β-arrestin mutant did not affect internalization or recycling of the receptor. Moreover, GnRH activation of the receptors did not induce mobilization of β-arrestin protein to the plasma membrane. A recent study by Heding and co-workers (2000) evaluated the role of β-arrestin and/or dynamin in rat GnRH receptor internalization (Heding A et al., 2000). COS-7 cells, transfected with the rat GnRH receptor gene, did not undergo β-arrestin dependent internalization. However, addition of the C-terminal tail of the TRH receptor to the C-terminus of the rat GnRH receptor resulted in a β-arrestin-dependent internalization. These data suggest that the determinants for β-arrestin-dependent internalization are located within the carboxy terminal domain of the TRH receptor, which is not present in mammalian GnRH receptors. Interestingly, similar to the TRH and other G protein-coupled receptors, non-mammalian GnRH receptors contain an intracellular C-terminal domain and these receptors undergo rapid internalization as compared to mammalian GnRH receptors (Pawson AK et al., 1998). These data suggest that β-arrestin-dependent internalization may confer rapid internalization in those species with a cytoplasmic carboxy terminal domain. Heding and co-workers (2000) also evaluated the role of Dynamin in the process of GnRH receptor internalization. Co-expression of the rat GnRH receptor with dominant negative dynamin (DynK44A) mutant impaired internalization resulting in decreased endocytotic rate constants. However, only a partial inhibition of internalization was noted suggesting more than one pathway for endocytosis. Therefore, although the mammalian GnRH receptor undergoes internalization, it may be through a β-arrestin independent and partially dynamin-dependent pathway.

After internalization, receptors can either be sorted into endosomes for recycling back to the plasma membrane or may become degraded within lysosomes (Koenig JA and Edwardson JM, 1997). Free GnRH receptor appears at the cell surface after internalization has been completed. Treatment with the protein synthesis inhibitor, cyclohexamide, did not inhibit the appearance of un-occupied receptor at the cell surface suggesting that the new receptors resulted from the recycling of previously internalized receptor. In contrast, Starling and co-workers (1988) described an increase in cell surface GnRH receptors, which was sensitive to inhibitors of microtubule function, protein synthesis, or inhibitors of energy metabolism (Starling L et al., 1988). Thus, the increase in concentration of GnRH receptors at the cell surface, following GnRH-induced internalization, may be due to recycling as well as new receptor synthesis.

Interestingly, comparative studies using image-intensified microscopy and pituitary cell cultures showed that inhibition of receptor-ligand complex patching (aggregation along cell surface) and capping (endosomes formation) did not affect GnRH-stimulated LH release (Conn PM and Hazum E, 1981). These observations suggest that patching, capping and subsequent internalization of the GnRH receptor-ligand complex may not be necessary for GnRH-induced LH release. However internalization of the ligand-receptor complex may participate in other cellular mechanisms such as desensitization and downregulation.

Ligand-receptor complex internalization represents one of several cellular responses to agonist for mediation of signal attenuation. Mechanisms of signal attenuation include endocytosis, desensitization, and downregulation. Endocytosis is defined as the movement of receptor from the cell surface to an internal compartment. Downregulation refers to loss of function due to the decline in receptor concentration secondary to agonist-induced endocytosis. In contrast, desensitization is the loss of function, which may be due to uncoupling of receptor from G proteins and/or internalization of receptor. Desensitization may be either homologous (dependent on ligand-bound receptor) or heterologous (independent of ligand-bound receptor). Agonist-induced receptor internalization/endocytosis contributes to homologous desensitization by depleting the cell surface of high-affinity receptors. Moreover, the potential pathways for homologous desensitization in G protein coupled receptors involve not only changes in receptor number but also in phosphorylation of the receptor and modifications of the heterotrimeric G protein associated with the receptor. Desensitization of G protein-coupled receptors, such as β-adrenergic receptors and rhodopsin, appears to be mediated through phosphorylation of key residues within the cytoplasmic carboxy terminal tail (Lorenz W et al., 1991). Additionally, other G protein-coupled receptors, such as the α_{2a}-

adrenergic receptors, undergo rapid desensitization via phosphorylation of residues within the third intracellular loop (Liggett SB et al., 1992). Phosphorylation of these residues results in binding of β-arrestins to the receptor and inhibition of the association between the receptor and G proteins. Although mammalian GnRH receptors lack an intracellular carboxy terminus, rapid desensitization and receptor internalization in pituitary cells exposed to GnRH has been documented (Hazum E et al., 1980). The lack of a cytoplasmic C-terminus and key residues within the third intracellular loop in the mammalian GnRH receptor has resulted in research regarding the mechanism of desensitization in the GnRH receptor of mammals. However, the mechanisms of GnRH receptor desensitization have yet to be completely elucidated.

Agonist (GnRH) occupancy of the GnRH receptor stimulates the exchange of GDP for GTP on its respective heterotrimeric G-protein. This in turn allows for the α-subunit of the heterotrimeric G-protein to regulate effector proteins. In addition to promoting signal transduction, the α-subunit maintains intrinsic GTPase activity so as to hydrolyze GTP to GDP, hence stopping signal transduction. The intrinsic GTPase activity of the α-subunit can be enhanced by an effector molecule such as PLC or by GTPase activating proteins, known as regulators of G-protein signaling (RGS). The RGS proteins in essence limit the half-life of activated α-subunits thus contributing to homologous desensitization (Watson N et al., 1996). Neill and co-workers (1997) co-expressed the rat GnRH receptor and RGS proteins (RGS3) in COS-1 cells and demonstrated a significant suppression of GnRH-induced IP_3 production (Neill JD et al., 1997a). Furthermore, RGS3 was shown to bind with $G_{q\alpha}$ protein in a model *in vitro* system. Northern and western blot analysis revealed expression of RGS3 mRNA and protein in the murine gonadotrope cell line (αT3-1). The authors concluded that a potential role might exist for RGS3 and other RGS in regulating GnRH receptor desensitization.

In addition to activating the heterotrimeric G-protein, the binding of GnRH to its receptor activates the hydrolysis of membrane-bound phosphatidylinositol, via phosphoinositidase C, resulting in the generation of diacylglycerol and inositol phosphates, which in turn activate protein kinase C (PKC) and increases intracellular Ca^{2+} concentrations (these pathways will be discussed in detail later) (Naor Z, 1990). Therefore, studies regarding the mechanism(s) of receptor desensitization have involved the potential desensitization of phosphatidylinositol hydrolysis, reduction in PKC activity, and inhibition of the ligand-induced cytosolic rise in Ca^{2+} levels. McArdle and co-workers (1995) reported that GnRH-stimulated desensitization did not occur via desensitization of phosphatidylinositol hydrolysis. (McArdle CA et al., 1995). GnRH treatment of αT3-1 cells resulted in a linear increase in total IP production and although there was a decline in IP-response following GnRH pretreatment, it was attributed to almost 50% reduction in cell surface GnRH receptor concentration following GnRH pretreatment. Cassina and co-workers (1997) reported similar declines in surface receptor number, in cultured rat pituitary cells treated with GnRH for 40 to 50 minutes (Cassina MP et al., 1997). Anderson and co-workers (1995) also demonstrated a linear relationship between GnRH exposure and rise in IP production (Anderson L et al., 1995). Moreover, short-term pretreatment with GnRH did not desensitize the IP response to GnRH stimulation. This study was performed in two different cell lines (αT3-1 and HEK-293) to eliminate the effect of a cell line. In contrast, Neill and co-workers (1977) demonstrated agonist-induced desensitization of IP_3 production in COS-1 cells transfected with an epitope-tagged version of the rat GnRH receptor (Neill JD et al., 1997b). A linear increase in intracellular IP_3 concentrations was noted during the first 30 seconds after beginning GnRH agonist treatment; however from 30 to 60 seconds, there was a decline in IP levels that leveled off after 180 seconds. Furthermore, a 5-minute pretreatment of COS-1 cells with GnRH induced near complete desensitization of IP_3 production. There was no evidence of GnRH receptor phosphorylation during the 5-minute pretreatment. Importantly, however, these studies were done in a heterologous system that might explain the different results attained in αT3-1 cells.

In contrast to IP production, Anderson and co-workers did describe a GnRH-stimulated Ca^{2+} response which was desensitized in both αT3-1 and HEK-293 cell lines (Anderson L et al., 1995). Similarly, McArdle and co-workers (1995) reported that the GnRH-pretreatment caused a significant decline in Ca^{2+} response to subsequent GnRH challenge (McArdle CA et al., 1995). GnRH pre-treatment affected both the spike phase response (mobilization from intracellular IP_3-sensitive Ca^{2+} stores) and the plateau phase response (mobilization of extracellular Ca^{2+} via PKC-sensitive channels) of Ca^{2+} to GnRH treatment. The desensitization of the Ca^{2+} spike response was rapid (10-20 min) in onset whereas the recovery was slow (4-6 h). Moreover, in a study evaluating the factors that might be important in restoration of the desensitized gonadotrope, Gorospe and Conn (1988) demonstrated that recovery from desensitization might involve functional recovery of the voltage sensitive Ca^{2+} channels (Gorospe WC and Conn PM, 1988). The authors of these studies concluded that GnRH receptor desensitization might occur through several mechanisms, including a decline in surface receptor number and a reduction in ligand-stimulated intracellular Ca^{2+} mobilization.

The apparent lack of GnRH-induced IP desensitization in homologous cell lines expressing the mammalian GnRH receptor is unique compared to other G protein-coupled receptors. This may be due to the absence of a cytoplasmic terminal tail. The intracellular C terminus of most G protein-coupled receptors has been shown to be functionally important for G protein coupling (Cotecchia S et al., 1990), agonist-induced receptor internalization (Fukushima Y et al., 1997), and or Ser/Thr phosphorylation-mediated desensitization (Fukushima Y et al., 1997). Although mammalian GnRH receptors are lacking a C-terminal tail, receptor desensitization and internalization have been described suggesting a different mechanism from that used by other G protein-coupled receptors. In an attempt to better understand these differences, several studies have been performed using chimeric constructs of the mammalian GnRH receptor. Lin and co-workers (1998) added the catfish 51-amino acid intracellular C terminus to the rat GnRH receptor. This chimera bound to GnRH with the same affinity and significantly increased the surface receptor number (Lin X et al., 1998a). Additionally, inclusion of the catfish C-terminal domain resulted in an altered pattern of receptor regulation from biphasic down-regulation and recovery to monophasic downregulation of the rat GnRH receptor. Conn and co-workers first characterized the biphasic regulation of mammalian GnRH receptors by GnRH (Conn PM et al., 1984). In their study, mammalian GnRH receptors initially under went down-regulation (0.5-4 h post treatment) followed by upregulation of the receptor (9 h post treatment). This biphasic phenomenon has been described in wild type gonadotropes as well as cell transfected with mammalian GnRH receptor genes (Conn PM et al., 1984).

A similar study comparing the chicken and human GnRH receptor reported that receptor-mediated internalization of GnRH agonist was significantly faster for the chicken compared to the human receptor (Pawson AK et al., 1998). Furthermore, truncation of the chicken receptor such that the C-terminus was removed resulted in a rate of receptor internalization that was not different from that of the human GnRH receptor. Willars and co-workers (1999) demonstrated that the rat GnRH receptor does not undergo agonist-dependent phosphorylation (Willars GB et al., 1999). Furthermore, chimera construction by the addition of the C-terminus from the African catfish GnRH receptor resulted in a significant increase in agonist-dependent phosphorylation. Similar data, reported by Heding and co-workers (1998), demonstrated that chimera constructs involving the incorporation of the cytoplasmic tail from the rat TRH receptor into the rat GnRH receptor resulted in rapid desensitization of IP production and increased internalization rates (Heding A et al., 1998). These studies suggest that an alternative mechanism from that used by nonmammalian receptors may be present in mammalian GnRH receptors. Moreover, the presence of an intracellular carboxy terminal domain in nonmammalian GnRH receptors raises the question of the evolutionary significance and physiological importance of the absence of the C-terminal domain in mammalian GnRH receptors.

In addition to receptor internalization and desensitization, downregulation of the GnRH receptor is an important feature of signal attenuation. Downregulation can be defined as a time-dependent reduction of receptor density in intact cells. GnRH receptor downregulation has been described in humans, primates, sheep and rats (Dieschke DJ et al., 1970; Santen RJ and Bardin CW, 1973; Nett TM et al., 1981; Huckle WR et al., 1989). Although it is understood that downregulation occurs due to continuous exposure to GnRH, the mechamism(s) of GnRH receptor downregulation are not well defined. Potential explanations for the reduction in receptor number include, a decline in receptor recycling, gene transcription or mRNA translation. A reduced rate of receptor recycling may result from ligand-promoted change of receptor sorting from the default endosomes-plasma membrane-recycling pathway to the endosomes-lysosome degradation pathway. Although internalization of GnRH receptor has been well documented, downregulation owing to changes in rate of receptor recycling have not been reported. In contrast, downregulation of the receptor due to suppression of gene transcription and/or translation have been described. A study done by Cheng et al., (2000) demonstrated a dose- and time-dependent decline in human GnRH receptor promoter activity after 24-h treatment with a GnRH analog or phorbol 12-myristate 13-acetate (TPA) (Cheng KW et al., 2000). Depletion of PKC or blockage of PKC by inhibitors of PKC reversed the downregulation of the human GnRH receptor promoter. Moreover, progressive 5' deletion of the human GnRH receptor gene identified a region of the gene responsible for the agonist-induced downregulation. This region included an AP-1 binding site that was nearly 1000 base pairs upstream from the translational start site. The authors of this study concluded that downregulation of the human GnRH receptor might be partially due to a PKC-dependent pathway which works via AP-1 binding sites within the 5' promoter region, resulting in suppression of gene transcription. A reduction in gene transcription would contribute to the decline in available cell-surface GnRH receptors hence promoting downregulation.

Similar data were described in αT3-1 cells. Time- and dose-dependent reductions in the level of receptor mRNA were observed after treatment of αT3-1 cells with GnRH analogs (Mason DR et al., 1994). In this same study, GnRH receptor concentration at the cell surface fell by nearly 50% after 2h treatment with 10^{-8} M GnRH analog. This suggests that a component of downregulation in αT3-1 cells is due to a decline in cell-surface receptors that is not compensated for by receptor recycling. In addition to the decline in GnRH receptor mRNA levels, studies in αT3-1 cells have also indicated decreased translation to be a component of GnRH receptor downregulation. A 40% reduction in the ability of RNA to direct synthesis of functional GnRH receptors was noted in αT3-1 cells after being exposed to GnRH for 24 h (Tsutsumi M et al., 1995). In this study, GnRH receptor mRNA levels were not altered with GnRH treatment suggesting that the downregulation of GnRH receptors in αTR-1 cells may be due in part to decreased translational efficiency. In addition to changes in gene regulation, continuous exposure of αT3-1 cells to GnRH resulted in cellular downregulation of both $G_{q\alpha}$ and $G_{11\alpha}$ (Shah BH and Milligan G, 1993). Therefore, although the mechanism(s) of GnRH receptor downregulation have not been completely described, evidence suggests that reduction in the rate of receptor recycling, gene transcription, and translational efficiency might contribute to downregulation.

Receptor Binding Kinetics

In order to study the interaction between a hormone (D) and its receptor (R), saturation analyses are performed and equilibrium constants are estimated. These analyses include studies with intact cells or tissue preparations and involve graphical and/or mathematical derivations. Regardless of the *in vitro* conditions (intact cells vs.

tissue preparations), saturation analyses are dependent upon the availability of radiolabelled ligand with high specific activity and its interaction with a single population of homogenous binding sites (receptors). Furthermore, the binding between radioligand and receptor should be saturable based on the existence of a finite number of receptors available in the biological preparations. Once the saturation of receptor occurs, an increase in radioligand will not result in an increased response (receptor binding). Prior to calculating rate constants, the saturability must be determined. To assess saturability, incubations are performed with increasing concentrations of radioligand and fixed amounts of receptor (cells/tissue preps.). Graphical representation of saturable binding plotted as the concentration of ligand-receptor complex (DR) versus free ligand concentration (D) produces a rectangular hyperbola (Figure 1-1).

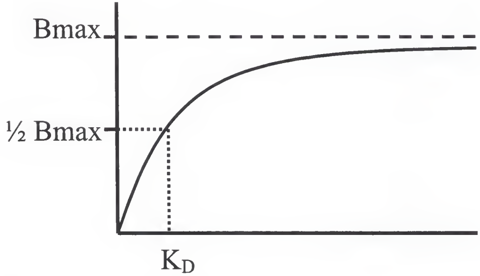

Figure 1-1. Saturation binding curve

$$D + R \xrightleftharpoons[k_2]{k_1} DR$$

Figure 1-2

The horizontal asymptote or concentration of (DR) at which the graph plateaus represents maximum binding (Bmax) of ligand to receptor and the reaction is said to be saturated. The reaction involving binding of the ligand with its receptor is a reversible reaction and behaves via the mass action law (Figure 1-2).

$$\frac{[DR]}{[D][R]} = \frac{K_1}{K_2} = K_A, M^{-1}$$

Figure 1-3

The abbreviation, D, is the ligand, R is the receptor, DR is the receptor-ligand complex, K_a is the association rate constant and K_d is the dissociation rate constant. Some publications list K_a and K_d as K_1 and K_2, respectively. At equilibrium, the rate of association = rate of dissociation and the ratio of receptor-ligand complex to the reactants is equal to the Equilibrium Association Constant (K_A) (Figure 1-3) An alternative method

$$\frac{[D][R]}{[DR]} = \frac{K_2}{K_1} = K_D, M$$

Figure 1-4

for expressing the affinity of the receptor for ligand in molar concentration is the Equilibrium Dissociation Constant (K_D). The K_D represents the concentration of hormone that half-maximally occupies the receptor at equilibrium and is equal to the ratio of reactants to receptor-ligand complex (Figure 1-4). The Equilibrium Dissociation Constant (K_D) is commonly used to report the affinity of a receptor for its ligand. An increase in K_D reflects a decrease in receptor affinity for the ligand whereas a decrease in K_D indicates an increase in affinity. The K_D can be predicted from the rectangular hyperbola representing the concentration of ligand-receptor complex (DR) versus increasing free ligand concentration (D) in a saturable reaction. The horizontal asymptote or concentration of (DR) at which the graph plateaus represents maximum binding (Bmax) of ligand to receptor and the reaction is said to be saturated. However as an asymptote, theoretically Bmax can only be truly achieved at a finite concentration of free ligand (D). At 50% of Bmax (concentration of ligand-receptor complex (DR)) the concentration of free ligand (D) is equal to the $K_{D.}$

Due to technical limitations occurring in some radioligand binding studies linear transformations of binding data are preferred for estimation of receptor affinity and receptor density. This type of transformation is advantageous because estimation of the K_D value and receptor density (Bmax) is made simple by calculating the slope of the line and its *Y* and *X intercepts*. However, several assumptions are inherent to the interpretation of these linear transformations. First, the hormone-receptor interaction is a simple, reversible reaction that obeys mass action law. Secondly, prior to estimating receptor affinity and receptor density the reaction must have reached equilibrium. Finally, the calculations of bound (DR) and free (D) radioligand must be accurate. Several linear

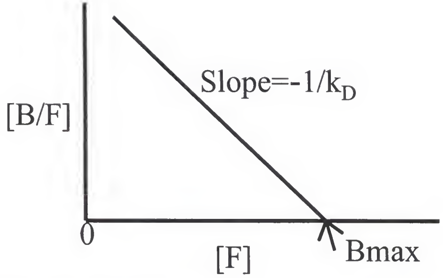

Figure 1-5 Scatchard plot analysis

transformations are routinely utilized including the Hill plot, the Double-reciprocal plot, and the Scatchard plot. By far, the Scatchard plot represents the most common linear transformation used for estimating the K_D value and receptor density. The Scatchard plot is represented graphically by plotting (DR)/(D) on the Y-axis versus (DR) on the X-axis, or Bound/Free (*B/F*) versus Bound (*B*) (Figure 1-5).

If the plot of *B/F* versus *B* represents a single, high affinity ligand binding with a single population of receptors the graph will be that of a straight line with a negative slope. This type of plot is consistent with a saturable system exhibiting cooperative behavior. The slope of the line = $-1/K_D$ or $-K_A$ and the X-intercept (Y=0) is an estimate of the receptor density (Bmax). Occasionally, linear transformation of saturation data does not transform to a linear plot. Nonlinear Scatchard plots can be either concave downward or concave upward. A physiologic explanation for concave downward Scatchard plot is that the receptor to which the ligand binds demonstrates positive cooperativity. This type of cooperativity occurs because the affinity of the receptor increases with increasing occupancy of receptor (bound fraction). This may be due to the binding of ligand to a single receptor possessing multiple binding sites for ligand or from receptor-receptor interactions provoked by binding of ligand to one of the receptor molecules. Alternatively, the nonlinear Scatchard plot may be a concave upward graph typical of negative cooperativity and indicative of a receptor affinity that declines with increasing occupancy of receptors. Additionally, the presence of two class of receptors, one of high affinity and one of low affinity will resemble the situation of negative cooperativity. This occurs because the ligand will bind with the high-affinity receptors first and will only bind to the low-affinity receptors at high ligand concentrations. This curvilinear Scatchard plot may be resolved into two straight lines allowing for estimation of K_D values and receptor density for each receptor population.

Scatchard plot and other similar types of saturation analyses allow for the estimation of receptor qualities (K_D, K_A, Bmax, etc) that are based on the assumption of a static environment such that the receptor remains unchanged even after binding with its ligand. However, this assumption is not valid when one considers that the cell plasma membrane is constantly changing in that receptors are being synthesized, internalized, recycled or degraded. More importantly, these cellular activities are typically in response to ligand binding. Therefore, ligand-binding assays are performed to evaluate the behavior of receptors following ligand stimulation and hence better characterize the receptor. Similar to saturation analyses, ligand-binding assays allow for the calculation of different rate constants which characterize receptor behaviors, such as the rate of recycling and internalization. Biochemical techniques are commonly used to assess receptor behavior by quantifying the cell surface receptor density versus the intracellular receptor-ligand complex in intact cells. One such technique includes determining the accessibility of hydrophobic versus hydrophilic ligands to receptors in intact cells. This method is based on the use of small, radiolabelled molecular weight ligands that bind to the plasma membrane receptors and increase the hydrophobicity such that the receptor-ligand complex passes into the cell. This approach is appropriate only when receptor binds to small molecular weight ligands. However many ligands are polypeptides which are too large to diffuse across the membrane. An alternative approach involves protease-resistant ligand binding assays as a measure of internalized receptor-ligand complexes. This technique takes into account the fact that protease treatment of cells rapidly hydrolyzes polypeptide hormones located on the cell surface whereas internalized proteins are spared from protease activity. Alternatively, cell surface bound radioligand can be dissociated by treating cells with an acidic solution. Haigler and co-workers (1980) demonstrated that surface-bound radioligand could be dissociated by lowering the extracellular pH. The total surface-accessible binding is estimated from the amount of radioligand removed during the acid wash. This procedure is performed at 4^0 C to prevent receptor population turnover and/or degradation. After removing the surface bound radioligand, the cell membrane is disrupted with a detergent and the intracellular receptor-ligand complex concentration is estimated by measuring the radioactivity released from within the cell. The concentration of intracellular radioligand represents radioligand-receptor complex plus radioligand, which has dissociated from the receptor. Because the radioligand tends to dissociate from the receptor complex once the ligand-receptor complex is internalized, it becomes difficult to document the path of the internalized receptor. A percentage of internalized receptors may be degraded within lysosomes and others may recycle to the plasma membrane. Several approaches exist for determining the rate of receptor recycling. The general strategy involves 4 criteria: First, the time it takes for the rate of receptor-ligand complex internalization to reach steady state must be determined. Second, after reaching steady state, all remaining unbound receptors must be inactivated (protease treatment). Third, time must be permitted for the reappearance of receptors on the cell surface. Finally, the reappearance of receptors must be scrutinized to distinguished between receptor recycling and the presence of new receptors on the cell surface. To distinguish between the appearance of new receptors and recycled receptors protein synthesis inhibitors such as, cyclohexamide, have been used. However, cyclohexamide is not specific for the GnRH receptor protein but rather inhibits the translation of all RNA. Hence, one limitation to the use of protein synthesis inhibitors is that inhibiting cellular protein synthesis may alter the biomechanics of receptor internalization and/or recycling resulting in misleading data. Additionally, it remains possible that the reappearance of surface-bound receptors may result from pre-existing pools of receptors that moved from the cytoplasm to the cell surface, following ligand stimulation.

Once the above mentioned criteria are satisfied, assessment of receptor recycling can be performed by calculating rate constants for receptor turnover using steady state mathematical analysis. The rate constants of interest include the endocytotic rate constant (K_e) and the rate of receptor recycling (V_R). The endocytotic rate constant is a cellular constant that defines the probability of an occupied receptor being internalized in 1 min at 37^0 C. Wiley and Cunningham (1982) proposed a mathematical model for estimating K_e. In their model, the concentration of ligand-receptor complex (intracellular) over surface receptor complex is plotted versus time. According to Wiley and Cunningham, the slope of the plot is equal to the rate of receptor endocytosis (K_e). Likewise, the rate of receptor recycling (V_R) can be estimated by a mathematical derivation. Once the K_e is determined, it is combined with the estimation of receptor density (Bmax) in order to calculate V_R. Therefore, as the number of receptors on the surface increases and/or the rate of endocytosis increases, the rate of receptor recycling also increases (Wiley HS and Cunningham DD, 1982).

GnRH Receptor Second Messenger System(s)

GnRH stimulates multiple actions in the gonadotrope. Determination of which actions are components of the second messenger system is dependent on three criteria. First, GnRH should stimulate a change in the effector molecule, such as an increase or decrease in cellular concentration. Second, activation of the effector independent of GnRH-induced activation should elicit an equivocal response as GnRH stimulation. Third, removal of the effector molecule should block the action of GnRH. GnRH stimulation of the GnRH receptor sets in motion a signal transduction process that involves activation of bound G-proteins, hydrolysis of phosphatidylinositol producing diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP_3), calcium mobilization, activation of protein kinase C, and activation of mitogen-activated protein kinases. These second messenger effectors are critical for eliciting an appropriate gonadotropic response to GnRH. Additionally; these effectors contribute to the regulation of the GnRH receptor through receptor desensitization and downregulation.

Cloning and sequencing of the GnRH receptor resulted in identification of the GnRH receptor as a member of the G protein-coupled receptor family. Guanyl nucleotide binding proteins (G proteins) are typically made up of heterotrimeric GTP-binding proteins, which dissociate upon ligand binding into α- and βγ-subunits (Spiegel AM et al., 1992). In the inactive state, the heterotrimeric complex is bound to GDP however once the receptor is activated by the ligand, the GDP molecule is exchanged for GTP. At this point the α-subunit remains bound to GTP and dissociates from the βγ-subunits. The α-subunit associates with different effector elements to propagate the signal, whereas the βγ-subunits typically remain together. In addition to the binding site for GTP, the α-subunit can hydrolyze GTP to GDP. Following activation and binding to GTP, the α-subunit converts GTP to GDP allowing for the α-subunit to re-associate with the βγ-subunits thus inactivating the G protein complex. Molecular cloning has revealed that α-subunits are encoded by at least 15 different genes, and β- and γ-subunits by 4 and 8 genes, respectively (Spiegel AM et al., 1992). Most G protein α-subunits contain one or two sites for nicotinamide-adenine dinucleotide (NAD)-dependent adenosine diphosphate (ADP)- ribosylation. This covalent modification is catalyzed by two distinct bacterial toxins, pertussis toxin and cholera toxin (Gilman AG, 1989). Several G proteins α-subunits ($G_{t1\alpha}$, $G_{t2\alpha}$, $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{0\alpha}$) are sensitive to the pertussis toxin, a toxin that catalyzes the ADP-ribosylation of the α-subunit and prevents interaction between the G protein heterotrimer and the receptor. These G proteins are responsible for regulating adenyl cyclase and the channels for K^+ and Ca^{2+} ions. In addition to pertussis toxin-sensitive G proteins, there exist G protein α-subunits that are not affected by pertussis toxin. These include $G_{s\alpha}$, $G_{olf\alpha}$, $G_{q\alpha}$, $G_{11\alpha}$, $G_{12\alpha}$, $G_{13\alpha}$, $G_{14\alpha}$, and $G_{15/16\alpha}$. These G proteins activate adenylyl cyclase and phospholipase C (PLC), which in turn catalyze the hydrolysis of phosphatidyl inositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Spiegel AM et al., 1992). Another bacterial toxin used to study G protein α-subunit function is cholera toxin. This toxin incorporates ADP-ribose into the α-subunit and causes persistent activation by blocking GTPase activity.

In murine αT3-1 cells the G protein α-subunits associated with the GnRH receptor have been identified as members of the $G_{q/11\alpha}$ family (Hsieh KP and Martin TFJ, 1992). Furthermore, GnRH activation of these cells involves initiation of the PLC cascade, which is dependent on receptor coupling to $G_{q\alpha}$, $G_{11\alpha}$, or both. Continuous exposure of αT3-1 cells to GnRH resulted in cellular downregulation of both $G_{q\alpha}$ and $G_{11\alpha}$ which may be a component of the desensitization of the GnRH receptor following sustained GnRH treatment (Shah BH and Milligan G, 1993). Recent work done by Grosse and co-workers (2000) suggested that CHO or COS-7 cells transfected with the human GnRH receptor, in addition to αT3-1 cells, are dependent on $G_{q/11\alpha}$ proteins (Grosse R et al., 2000). These data were corroborated by treating all cell lines with pertussis toxin, which resulted in no change in GnRH-induced activation. In contrast, work done in rat pituitary cell cultures and human tumor cell lines has identified the $G_{i\alpha}$ and $G_{s\alpha}$ proteins as the α-subunits involved in GnRH-activation of the GnRH receptor (Hawes BE et al., 1993). Pertussis toxin pre-treatment of rat pituitary cell cultures resulted in an inhibition of GnRH-stimulated inositol phosphate (IP) production, implicating the involvement of $G_{i\alpha}$ subunits. Additionally, pretreatment of rat pituitary cells with cholera toxin resulted in a potentiated GnRH-stimulation of LH release, thus implicating the $G_{s\alpha}$ subunit. Similar results were obtained studying lactotrope-derived cell lines expressing the GnRH receptor (GGH_3) (Stanislaus D et al., 1998).

Activation of PLC in gonadotropes results in the hydrolysis of various plasma membrane-bound phosphatidyl inositols, PIP_2 being the primary substrate for GnRH activation (Naor Z et al., 1986). GnRH stimulation of cultured rat pituitary cells resulted in a rapid and progressive increase in the formation of $Ins(1,4,5)P_3$ (IP_3) (Morgan RO et al., 1987). Similarly, in murine αT3-1 cells, GnRH activation causes a significant rise in IP_3 and IP_2 levels (Horn F et al., 1991). Intracellular concentrations of IPs increased within 30 seconds following exposure of αT3-1 cells to GnRH and continued to rise until reaching maximum levels after 20 minutes (Anderson L et al., 1993). These studies indicated that GnRH-induced IP_3 production was biphasic, with an early, fast rise followed by a subsequent slower rise. Furthermore, data from these studies satisfied the first of three criteria for a second messenger effector, being that GnRH stimulated a change in concentration of IP_3 production. Confirmation of IP_3 as a second messenger effector in activated pituitary cells required treatment of cells with an IP_3 inhibitory substance as well as exposing cells to IP_3 or an IP_3-like substance. Treatment of rat pituitary cells with a PLC inhibitor resulted in a decline in IP_3 production, Ca^{2+} mobilization, and LH response (Hawes BE et al., 1992). Furthermore, addition of IP_3 to individual gonadotropes, via a patch-clamp, pipet resulted in the mobilization of Ca^{2+} in a manner that was not different from that of GnRH-activated gonadotropes (Stojilkvic SS et al., 1993). Thus the hydrolysis of inositol phosphates, yielding IP_3, appears to satisfy the requirements for a second messenger effector in that, GnRH-activation causes the synthesis of IP_3, removal or inhibition of IP_3 reduces the GnRH-activated cellular response, and IP_3 treatment activates pituitary gonadotropes in a GnRH-like manner.

The initial product of hydrolysis, IP_3, binds to its intracellular receptors and induces rapid increase in intracellular Ca^{2+} concentrations. Samli and Geschwind (1968) demonstrated the importance of Ca2+ mobilization for biological action of pituitary gonadotropes. Chelation of Ca^{2+}, in extracellular medium, completely blocked hypothalamic extract-stimulated LH release from pituitary tissue (Samli MH and Geschwind II, 1968). Additionally, substances which increase intracellular stores Ca^{2+} concentration, such as ionophores and ion-bearing liposomes, were shown to release LH in a manner not different from that of GnRH (Marian J and Conn PM, 1979). When Ca^{2+} ionophores and GnRH were both added to cultured rat pituitary cells, the cells did not release more LH than with either secretogogue alone (Conn PM et al., 1979). In addition, treatment of pituitary cells with Ca^{2+} channel blockers, such as verapamil, resulted in a reduced GnRH-induced LH release (Marian J and Conn PM, 1979). These studies concluded that GnRH-stimulated, LH release required a minimum concentration of Ca^{2+} for optimal stimulation although basal production did not appear to be calcium dependent. A potential mediator of Ca^{2+} signal in gonadotropes is calmodulin. This intracellular factor binds Ca^{2+} and then alters the activity of intracellular enzymes and cytoskeletal proteins involved in the secretory process (Jennes L et al., 1985). Immunohistochemical studies revealed the presence of calmodulin in association with the GnRH receptor. Furthermore, treatment of gonadotropes with Ca^{2+} -calmodulin blocking agents, such as pimozide, resulted in a 50% inhibition of LH release in response to GnRH or Ca^{2+} ionophores (Conn PM et al., 1981). Treatment of cultured rat pituitary cells with pimozide did not inhibit the binding of GnRH with its receptor suggesting that its blocking effect on LH release was not at the receptor level. Moreover, GnRH- or ionophore-stimulated Ca^{2+} mobilization was not altered by pimozide treatment. The authors concluded that the inhibition of LH release due to pimozide was due to the inhibitory effect of pimozide on calmodulin binding with Ca^{2+}. Three major calmodulin-binding proteins have been identified within gonadotropes, including spectrin, calcineutrin, and caldesmon (Wooge CH and Conn PM, 1988). Caldesmon binds F actin in the presence of low Ca^{2+} concentration and calmodulin in the presence of elevated Ca^{2+} concentrations. Therefore, GnRH stimulation of Ca^{2+} mobilization, following receptor binding, may provide an environment suitable for calmodulin to interact with caldesmon. When caldesmon-actin interaction was blocked, the interaction between calmodulin and caldesmon was increased, as was the GnRH-stimulated release of LH (Janovick JA et al., 1991). These data support the proposed role for calmodulin in GnRH stimulation of gonadotropes.

More recent studies have contributed to a better understanding of the intracellular events, which facilitate Ca^{2+} mobilization within gonadotropes. The initial rise in intracellular Ca^{2+} levels is followed by external Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Naor Z, 1990). In both cultured gonadotropes and αT3-1 cells, the immediate increase in intracellular Ca^{2+} is extracellular Ca^{2+}- independent whereas the subsequent sustained phase is dependent on Ca^{2+} entry through the voltage-gated channels (Merelli F et al., 1992). Furthermore, the initial calcium response was complete within several seconds and was IP_3 -mediated due to release from intracellular stores (endoplasmic reticulum). The smaller secondary plateau phase lasted minutes and involved influx of extracellular calcium through PKC-sensitive channels (Naor Z, 1990). Therefore, the initial GnRH-induced hydrolysis of inositol phosphates appears to initiate the influx of Ca^{2+} from intracellular stores whereas the subsequent rise in intracellular Ca^{2+} appears to be mediated through the PKC-activation of Ca^{2+} channels. Based on the above-mentioned criteria for classifying second messenger effectors, calcium appears to play such a role in the GnRH-stimulated, calcium-dependent release of LH.

In addition to the production of IP_3, diacylglycerol (DAG) is produced from the hydrolysis of inositol phosphates. DAG is a co-activator of protein kinase C (PKC). GnRH stimulation of pituitary gonadotropes results in an immediate rise in concentrations of DAG, similar to that of IP_3 (Chang JP et al., 1988). A variety of isoforms exist for PKC including cPKC, nPKC, and aPKC (Nishizuka Y, 1992). These isoforms vary in that they are activated by different second messenger factors, which include Ca^{2+}, DAG, and phosphatidylserine. Studies performed in permeabilized pituitary cells indicated that cPKCα and cPKCβ stimulate LH release (Naor Z et al., 1989). The primary method of activation initiated by protein kinase C is that of protein phosphorylation. Treatment of pituitary cells with inhibitors of protein phosphorylation resulted in a marked reduction in GnRH-stimulated LH release suggesting that protein phosphorylation is positively involved in GnRH stimulation of gonadotropin release (Marantz Y et al., 1995).

Activation of PKC results in the translocation of PKC from the cytosol to the plasma membrane and the activation of voltage-sensitive Ca^{2+} channels. This has been documented in αT3-1 cells, in which GnRH stimulation resulted in translocation of PKC towards the plasma membrane (Horn F et al., 1991). Furthermore, treatment of pituitary cells with DAG resulted in activation of PKC similarly to that of GnRH activation, which was Ca^{2+} independent (Conn PM et al., 1985). In a similar study done by Naor and Eli

(1985), DAG and a potent phorbol ester both stimulated PKC activity even at low Ca^{2+} concentrations, indicating that PKC activation is calcium-independent (Naor Z and Eli Y, 1985). Studies using αT3-1 cells documented an increase in GnRH receptor gene promoter activity by phorbol ester (PMA), a potent PKC activator treatment that was not different from GnRH-induced promoter activity. Furthermore, pretreatment with specific phorbol ester inhibitors blocked the GnRH- and PMA-induced increase in promoter activity (White BR et al., 1999). Interestingly, activation of the GnRH receptor gene by either GnRH or PMA increased the mRNA expression of c-Jun, C-Fos, and JunB (Cesnajaj M et al., 1994). As discussed earlier, these nuclear proteins recognize an AP-1 binding site located on in the promoter region of the GnRH receptor. Simultaneous treatment of cultured rat pituitary cells with a calcium ionophore and a potent PKC activator (tumor promoter phorbol acetate, TPA) resulted in a synergistic response that was similar to the physiological response to GnRH (Naor Z and Eli Y, 1985). In contrast, work done in cultured ovine pituitary cells failed to demonstrated a PKC-dependent LH response to GnRH stimulation (Beggs MJ and Miller WL, 1989). Treatment of dispersed ovine pituitary cells with PMA plus GnRH stimulated more LH release than either one alone. Furthermore, exposure of cells to a PKC inhibitor only suppressed the PMA-stimulated LH release and not the GnRH-induced LH release. These data suggest that the stimulatory effect of GnRH on LH release may be mediated through more than one intracellular second messenger pathway and may be species specific.

In addition to regulating the mobilization of extracellular Ca^{2+} via voltage gated channels, activated PKC plays a role in stimulating cytosolic protein kinases, including the mitogen activated protein kinase cascade (MAPK), also known as extracellular signal-related kinases (ERKs). Several G protein-coupled receptors appear to activate MAPK through Ras-dependent and –independent pathways, and both Gα- and G$\beta\gamma$-subunits appear to be involved (Crespo P et al., 1994). Initiation of the MAPK cascade results in translocation of MAPK to the nucleus resulting in the MAPK-activation of certain transcriptional factors, such as Elk and c-Fos (Seger R and Krebs EG, 1995). Recent work by Han and Conn (1999) demonstrated GnRH activation of MAPK activity in GGH_3 cells (Han XB and Conn PM, 1999). GGH_3 cells are rat pituitary cells, derived from rat pituitary tumor cells (GH_3), which have been transfected and express the rat GnRH receptor gene. Furthermore, treatment of GGH_3 cells with phorbol ester (PKC-activator) resulted in phosphorylation and activation of MAPK. However, PKC depletion from GGH_3 cells prior to GnRH agonist treatment did not prevent MAPK activation. These data suggest a PKC-independent mechanism for MAPK activation in GGH_3 cells. Treatment of αT3-1 cells with either GnRH or TPA resulted in similar activation of MAPK. Moreover, downregulation of endogenous PKC activity suppressed GnRH- and TPA-induced MAPK activation in a similar manner (Naor Z et al., 1998). Interestingly, calcium ionophore treatment did not result in activation of MAPK, however chelation of Ca^{2+} resulted in a significantly reduced MAPK activation by GnRH and TPA. These results suggested that MAPK might be activated in PKC-dependent manner, with Ca^{2+} being necessary but not sufficient. Work done by Naor and co-workers (2000) further supported the activation of MAPK by GnRH demonstrating the GnRH-stimulation of four mitogen-activated protein kinase cascades by a PKC-dependent mechanism (Naor I et al., 2000).

In addition, MAPK phosphorylates phospholipase A_2 (PLA_2), resulting in activation of (PLA_2) and the subsequent release of arachidonic acid (AA) from cellular phospholipids plus the formation of eicosanoids (Naor Z, 1991). GnRH-stimulation of cultured pituitary cells has been shown to induce the release of AA and the formation of several 5-lipoxygenase products (Dan-Cohen H et al., 1992). Both AA and MAPK-induced transcriptional factors may play a role in GnRH-induced gonadotropin gene regulation such that GnRH provides a signaling sequence originating at the cell surface and terminating in gene expression in the nucleus (Ben-Menahem D et al., 1994).

New information regarding characteristics of the GnRH receptor is continually being discovered. Areas of particular interest include the mechanisms of signal attenuation, in particular receptor desensitization and downregulation. This is particularly true in the case of horses. As discussed throughout the literature review, horses appear to be insensitive to signal attenuation during exposure to continuous GnRH. Hence, the following studies were designed to better understand the apparent lack of signal attenuation in horses.

CHAPTER 2
CLONING, SEQUENCING, AND TISSUE EXPRESSION OF THE EQUINE GONADOTROPIN-RELEASING HORMONE RECEPTOR

Introduction

Gonadotropin-releasing hormone (GnRH) exerts its biological effects through binding to and activating the GnRH receptor on pituitary gonadotrope cells. The GnRH receptor is a member of the G protein-coupled receptor superfamily, which is characterized by seven transmembrane domains and association with $G_{q/11}$ class of G-proteins (Hsieh KP and Martin TFJ, 1992). Activation of the GnRH receptor requires pulsatile GnRH to maintain gonadotrope function such that the anterior pituitary of rats (Catt KJ et al., 1983), sheep (Nett TM et al., 1981), primates (Marshall JC et al., 1983) and humans undergo downregulation of GnRH receptors due to constant GnRH administration (Belchetz PE et al., 1978). Pulsatile GnRH release is necessary to maintain physiological function in these species and the endogenous pulsatility of GnRH and LH secretions have been described in primates (Carmel PW et al., 1976), rodents (Levine JE and Ramirez VD, 1980), and humans (Santen RJ and Bardin CW, 1973). Episodic secretion of LH and GnRH also has been described in horses by cannulating the cavernous sinus and simultaneously measuring GnRH and LH. Studies using this technique have demonstrated a close correlation between GnRH, FSH, and LH secretion (Alexander SL and Irvine CHG, 1987; Irvine CHG and Alexander SL, 1994b). However, unlike other species, horses appear to have a less definite pattern of GnRH release. One study reported very irregular episodes of gonadotropic secretion in horses (Evans JW et al., 1979). Other studies described gonadotropin secretory events in mares as “episodes” rather than “pulses” due to their lack of rhythmical nature (Irvine CHG, 1995).

Another technique used to simultaneously measure GnRH and LH in horses is push-pull perfusion of the anterior pituitary gland. Grubaugh and Sharp (1987) studied GnRH release during various times of the year in pony mares using this technique. The secretory pattern of GnRH was monitored at ten-minute intervals and best described as "irregularly episodic but not regularly pulsatile." Further, not all GnRH secretory events preceded a rise in LH. The study concluded that the distinct secretory events occurred with no apparent regularity and were not circhoral. In addition, GnRH secretion in horses has been described as being episodic, with secretory events occurring two to three times per 12 h during diestrus and increasing in episode frequency during estrus, becoming continuous (Alexander SL and Irvine CHG, 1987). In a more recent study, evaluation of LH secretion revealed a pulsatile pattern of release in ewes and a non-pulsatile secretion of LH in pony mares (Porter MB et al., 1997b). In summation, the pattern of GnRH and LH secretion in horses does not appear to be strictly pulsatile making it different from that of other species. Furthermore, these data suggest a lack of necessity for pulsatile GnRH in horses due to endogenously, non-pulsatile GnRH secretion in horses.

Studies done in our laboratory evaluating gonadotropic response to GnRH in pony mares have documented resistance by the equine gonadotrope to receptor desensitization or downregulation when continuously exposed to GnRH (Porter MB et al., 1997a); (Porter MB et al., 1997b). The mare’s response to continuous GnRH is distinct from other mammalian species, which typically respond to continuous GnRH exposure with a secretory pattern indicative of receptor downregulation. The maintenance of a gonadotropic response during continuous GnRH exposure in horses may involve, variations in GnRH receptor sequence which impart structural and/or functional differences, variations in GnRH receptor kinetics, or variations in signal attenuation.

The nucleotide sequence of the GnRH receptor has been published for several mammals including rats (Eidne KA et al., 1992), mice (Zhou W and Sealfon SC, 1994), ewes (Brooks J et al., 1993), cows (Kakar SS et al., 1993b), and pigs (Weesner GD and Matteri RL, 1994). A high degree of homology (>80%) has been reported across these GnRH receptors. Studies designed to introduce point mutations within the nucleotide sequence have shown that a single amino acid difference may be capable of altering the functional state of the GnRH receptor (Cook JV et al., 1993); (Arora KK et al., 1995); (Flanagan CA et al., 1997);(Cook JV and Eidne KA, 1997). Furthermore, although the GnRH receptor is a member of the G protein-coupled receptor family, mammalian GnRH receptors differ significantly from other G protein-coupled receptors. In particular, the mammalian GnRH receptor lacks a cytoplasmic carboxy-terminal tail. In addition, highly conserved amino acid residues present in most G protein-coupled receptors are not present in mammalian GnRH receptors.

Among the anterior pituitary cells, GnRH binding sites are located exclusively in three populations of gonadotropes. Those which express both LH and FSH and those which express only LH or FSH (Naor Z and Childs GV, 1986). However, Northern blot analyses and reverse transcriptase polymerase chain reaction (RT-PCR) have identified mRNAs encoding the GnRH receptor in extrapituitary tissue. Kakar and Jennes (1995) used RT-PCR to screen various human reproductive and non-reproductive tissues (Kakar SS and Jennes L, 1995). PCR products of expected size were identified in human liver, heart, skeletal muscle, kidney, placenta, and pituitary. Southern blot analysis was used to confirm the authenticity of the PCR products.

Information does not exist regarding the nucleotide sequence, amino acid sequence or tissue expression of the equine GnRH receptor; therefore the purpose of this study was to characterize the equine GnRH receptor through cloning and sequencing. Although a high degree of homology exists among the published sequences of mammalian GnRH receptors, determination of the equine GnRH receptor sequence might reveal distinct features, which could explain its refractoriness to desensitization and downregulation. In addition, availability of the equine GnRH receptor clone would provide a molecular tool for studying the *in vivo* and *in vitro* expression of the equine GnRH receptor.

Materials and Methods

The equine GnRH receptor was cloned and sequenced by screening an equine pituitary cDNA library that was constructed and supplied by Dr. Michael Wolfe (Kansas State Medical Center, Kansas, MO). The library consisted of the ZAP Express® EcoR I/ *X*ho I library manufactured by Stratagene Cloning Systems (La Jolla, CA) and prepared from equine anterior pituitary gland RNA.

Preparation of cDNA Probe

A cDNA probe, coding for a portion of the equine GnRH receptor, was used to screen the cDNA library. The probe was prepared as follows: Total cellular RNA was prepared from equine anterior pituitary glands by the TRIzol™ method (Life Technologies, Inc). Equine anterior pituitary gland tissue (100 mg) was homogenized briefly in 1 ml of TRIzol™ reagent (phenol and guanidine isothiocyanate solution).

Addition of chloroform followed by centrifugation, divided the solution into an aqueous phase, containing the RNA, and an organic phase. The aqueous phase was recovered and the RNA was precipitated from this solution with isopropanol. After precipitation, RNA was re-constituted in sterile water and total RNA quantified by measuring the absorbance at 260 nm.

RNA (5μg) was reversed transcribed to cDNA using Oligo dT primer and AMV Reverse Transcriptase, according to the protocol provided by the cDNA cycle kit™ (Invitrogen, San Diego California). Subsequently, the first strand cDNA was used as a template in Polymerase Chain Reaction (PCR) amplifications. PCR was performed using consensus primers designed from mouse and rat GnRH receptor sequences. The nucleotide sequence of the primers used in PCR were the following: sense 5'-CATGATGGTGGTG-ATYAGCCTSGA-3' and antisense 5'GAAGAAGTGATT-KACTGGMTCTGA-3'. The PCR conditions were optimized for the number of cycles performed and amount of cDNA template added such that the PCR products were in the logarithmic phase. Additionally, the pH and magnesium concentrations were optimized for the PCR reaction with the PCR Optimizer™ kit (Invitrogen, San Diego California). The PCR conditions were the following: Step 1) 95^0 C for 2 min, 2) 94^0 C for 1 min, 3) 55^0 C for 2 min, 4) 72^0 C for 3 min (repeat steps 2-4 for 33 cycles), 5) 94^0 C for 2 min, 6) 55^0 C for 3 min, and 7) 72^0 C for 7 min. A total of 0.5 μl (represents 1 μg of reverse-transcribed total RNA) of cDNA template was added to each reaction. Fifteen microliters of the 50 μl reaction mixtures from each sample were electrophoresed through a 1.5% agarose gel stained with ethidium bromide and subjected to a densitometric analysis.

The TA cloning kit™ from Invitrogen was used to ligate the PCR product to a plasmid vector (pCR™II vector). Ligation reactions were transformed into One Shot™ competent cells. Transformations were then streaked onto LB agar plates (containing ampicillin and X-gal) and incubated at 37 C overnight. Subsequently, the appropriate colonies were picked for plasmid isolation and sequencing. Sequencing of the cloned PCR products required isolation of ultra pure plasmid DNA in significant quantities (100-500ugs); therefore, Plasmid Mini- and Maxi-Prep kits (Qiagen Inc, Valencia, CA) were used. Sequencing of the cloned PCR product was performed by the DNA Sequencing Core of the Interdisciplinary Center for Biotechnology Research, at the University of Florida (Gainesville, Florida).

Screening cDNA Library

The equine pituitary cDNA library was screened with the $^{32-}$P labeled equine GnRH receptor DNA probe following the recommended protocol for the ZAP Express® EcoR I/ *X*ho I library (Stratagene). In brief, titration of the phage library (pfu/ml) was performed and approximately 1 X 10^6 plaques from the library were plated (50,000 pfu/plate) along with a desired concentration of *E. coli* XL1-blue. After 12 hr of incubation at 37^0C, nitrocellulose membranes (Schleicher and Schuell) were briefly placed onto each plate, in duplicate, to lift phage DNA. Nitrocellulose membranes were removed from plates, passed through a series of denaturing and neutralizing steps and baked at 80^0C for 2 hours. Subsequently, the nitrocellulose membranes were probed with $^{32-}$P labeled equine GnRH receptor cDNA (PCR product) prepared with Nick translation kit (Amersham pharmacia biotech). Pre-hybridization and hybridization were performed using ULTRAhyb™ buffer as recommended by the supplier (Ambion, Inc). The blots (nitrocellulose membranes) were exposed to x-ray film at -80 C for 24 hr and examined by autoradiography. Positive clones were identified, selected and purified to homogeneity via 3 successive screening processes (primary, secondary, and tertiary screenings). Excision of the pBK-CMV phagemid from the ZAP Express vector was performed with the aid of ExAssist® Interference-Resistant Helper Phage (Stratagene) and sufficient plasmid DNA for sequencing was recovered using Maxiprep kits (Qiagen). Six positive clones were purified and sequenced by the DNA Sequencing Core of the Interdisciplinary Center for Biotechnology Research, at the University of Florida (Gainesville, Florida).

Tissue Expression of Equine GnRH Receptor mRNA

Equine tissue samples collected included anterior pituitary gland, hypothalamus, cerebral cortex, olfactory lobe, liver, kidney, spleen, corpus luteum, and granulosa cells from pre-ovulatory follicles. All tissues were collected from the same pony mare except for the corpus luteum and granulosa cells, which were collected from separate pony mares. Tissues were rapidly frozen in liquid nitrogen and stored at -70^0C until the time of RNA isolation. Total RNA from tissues was prepared by the TRIzol™ method (Life Technologies, Inc), as described earlier. For Northern blot analysis, approximately 30 μg of total RNA were separated on 1.0% agarose-2.2 M formaldehyde gels and gels transferred to a nitrocellulose membrane by downward capillary transfer using the TurboBlot system (Schleicher & Schuell, Keene, NewHamshire). Nitrocellulose membranes were cross-linked by brief (60 sec) exposure to ultraviolet light and the membranes were baked for 2 hours at 80^0C. The prehybridization and hybridization of the membranes was done using ULTRAhyb™ Buffer (Ambion) and the $^{32\text{-}}$P-labelled equine GnRH receptor cDNA probe was prepared by following the protocol as provided by the Nick translation kit (Amersham Pharmacia Biotech). Following overnight hybridization with the $^{32-}$P-labelled equine GnRH receptor cDNA, the membranes were subjected to several low and high stringency washes. The blots were then examined by autoradiography. Densitometric analysis of the developed bands on the autoradiograms was performed to quantify GnRH receptor mRNA. The blots where rehybridized with $^{32-}$P-labelled equine house-keeping gene cDNA probe (GAPDH) and the densitometeric values representing GAPDH mRNA were used to adjust for differences in RNA loading.

Results

Equine Pituitary cDNA Probe

Sequencing of the cloned PCR product yielded 535 nucleotides (Figure 2-1) that retained a high (>84%) degree of homology with other published GnRH sequences (Table 2-1). There are 984 nucleotides in the GnRH receptor sequence of most mammals, with the exception of the mouse and rat (981), which are lacking a single amino acid. The cloned PCR product corresponded to approximately 54% of the open reading frame for the GnRH receptor. Moreover, BLAST alignment of the partial equine sequence with other full GnRH sequences indicated that the partial equine sequence corresponded to the distal half of the GnRH receptor of other mammals (Figure 2-2). Thus, the nucleotide sequence of the proximal or 5'-end of the equine GnRH receptor remained undetermined. The variation in sequence homology between most mammals and rodents was due to a deletion of 3 nucleotides in the mouse and rat GnRH receptor.

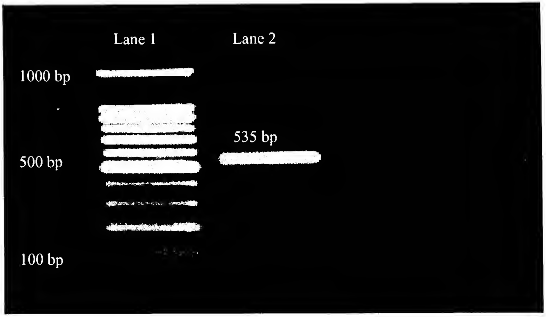

Figure 2-1. Polymerase chain reaction (PCR) was performed on 1.0 µl of cDNA produced from equine anterior pituitary tissue. The PCR product (15 µl) was loaded on a 1% agarose gel (lane 1) and a 100-nucleotide base pair ladder was loaded in lane 1. Primers used for PCR were designed based on regions of high homology between the mouse and rat GnRH receptor sequences. Gel electrophoresis displayed a PCR product of 536 base pairs in length.

Table 2-1. Sequence identity between partial equine GnRH receptor sequence (amino acid) and that of other mammals.

Species	**% Identity**
Ovine	93%
Bovine	93%
Porcine	92%
Human	92%
Murine	85%

```
Equine   1     catgatggtggtgatcagcctggaccgctcactggccatcacaaggcccctagctgtgaa 60
Ovine    429   .....................c........c...........c.....t.....a..... 488
Bovine   469   .....................c........g.....g.....c.a...t.....a..... 528
Human    414   ..............................c.....t.....g............t.... 473
Swine    408   ...............t..............g....................c........ 467
Murine   435   ...............t..............c...........tca......t.....ac. 494

Equine   61    aagcaacagcaagcttggacggtccatgattggcttggcttggcttctcagtagtatctt 120
Ovine    489   ...............c....a..t...............c.....c........c..... 548
Bovine   529   ....................a..t...............c.....c........c..... 588
Human    474   ............ag.c....a.......g.....c....c...a.c.........g.... 533
Swine    468   ...........g...c.......t...............c.....c........c..... 527
Murine   495   .................a..a...t.....ca..c....c...a.......c.t.g.... 554

Equine   121   tgctggaccacagttatacatcttccggatgatccatttagcagacagctccggacagac 180
Ovine    549   ........................tg...................tga...t........ 608
Bovine   589   .............c..........tg...................tga...t........ 648
Human    534   ...a.....................a.......t...c.............t........ 593
Swine    528   .........................a.........................t........ 587
Murine   555   ...a..............t......a........t.cc........g....t..g.cc.. 614

Equine   181   agaaggtttctctcaatgtgtaacacactgcagttttccacaatggtggcatcaagcctt 240
Ovine    609   t.........................................g................. 668
Bovine   649   t..............g..........................g................. 708
Human    594   .a...t..............................t....................a.. 653
Swine    588   ...........................tg............................... 647
Murine   615   ..tc---.....g........g..c........c........g...........g..... 671

Equine   241   ttacaattttttcaccttcagctgcctcttcatcatccctcttctcttcatgttgatctg 300
Ovine    669   ...t..c.......................................a.....c....... 728
Bovine   709   ...t..c.......................................a.....g....... 768
Human    654   ...t..c....................................t..a.....c....... 713
Swine    648   ...tg.c...........t..............t........c...a............. 707
Murine   672   c.....c............g...................c..c...a.....c.a..... 731

Equine   301   caatgcaaaaatcatcttcaccctgacaagagtccttcatcaggatccccacaaactaca 360
Ovine    729   ........................a.....g............................. 788
Bovine   769   ..................t.....a.....g............................. 828
Human    714   ............................c.g..............c......g....... 773
Swine    708   ...c...........g.....t.................g..............c..g.. 767
Murine   732   ......c...........tg.t..c..gc.............a..c..a.g......... 791

Equine   361   actgaatcaatccaagaacaatataccaagagctcggctgaggaccctaaagatgacggt 420
Ovine    789   .........g..................ca.............................. 848
Bovine   829   .........g..................c............................... 888
Human    774   .........g.......................a.......a...t.....a........ 833
Swine    768   g....................c......c........t.......t..g........a.. 827
Murine   792   g........g........t.....c.................a..g...........a.. 851

Equine   421   tgcatttgccacttcatttactgtctgctggactccctactatgtcctaggaatttggta 480
Ovine    849   .........g.......................g..............t........... 908
Bovine   889   .................................g..............t........... 948
Human    834   ............................................................ 893
Swine    828   .........tg.........t....................ta................. 887
Murine   852   c.....c..t..c..c...gtc.............................c........ 911

Equine   481   ttggtttgatcctgaaatgttaaacagggtgtcagatccagtcaatcacttcttc 535
Ovine    909   ...............c...g......................a............ 963
Bovine   949   ...............c...g......................a............ 1003
Human    894   ............................t.......c.....a............ 948
Swine    888   c...........c......g................................... 942
Murine   912   c...........a........g..............g.....g........     962
```

Figure 2-2. Consensus nucleotide sequence alignment (BLAST) of RT-PCR product (partial GnRH sequence) and other GnRH receptor sequences. Regions of homology are designated with a dot and non-homologous nucleotides are listed. Primer regions are in bold.

Screening of Equine Pituitary cDNA Library

Six clones that hybridized with the equine GnRH receptor cDNA probe were isolated from the equine pituitary cDNA library (Figure 2-3). All six of the clones were purified and sequenced. Analysis of the nucleotide sequences indicated that they were identical to each other and were composed of 1159 nucleotides (Figure 2-3).

The nucleotide sequence encodes a putative 328 amino acid protein consistent with that of the GnRH receptor. The equine GnRH receptor is more similar to the ovine, bovine, swine, and human receptor than to the rodent (mouse and rat) GnRH receptor (Table 2-2). Although there is a high degree (>85%) of homology between the equine GnRH receptor and that of other mammals, there are several amino acid residues within the open reading frame that are completely unique to the horse. These include Ser^{16}, Ala^{25}, His^{61}, Asn^{69}, and Phe^{225}. In addition there are several residues that are different from those in at least 75% of other mammalian GnRH receptors. These include Thr^{50}, Lys^{75}, Arg^{156}, and Leu^{314}. Similar to other GnRH receptors, the equine receptor contains potential sites for post-translational modification, which include N-linked glycosylation sites: four cysteine (Cys^{15}, Cys^{114}, Cys^{197}, and Cys^{201}) residues in the extracellular loops, and; multiple serine and threonine residues in the cytoplasmic domain that may play a role in regulatory phosphorylation.

Equine GnRH Tissue Expression

Tissue distribution of the equine GnRH receptor was determined using Northern blot hybridization. This analysis revealed receptor mRNA in equine pituitary tissue but not in other tissues (Figure 2-4) (hypothalamus, cerebral cortex, olfactory lobe, liver, kidney, spleen, corpus luteum and granulosa cells).

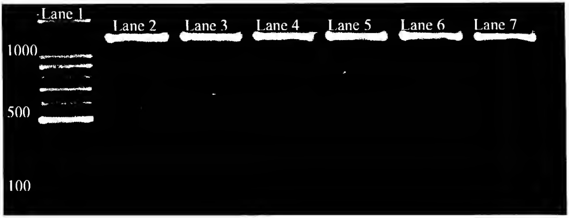

Figure 2-3. Gel electrophoresis of six clones (lanes 2-7) recovered during the screening of equine cDNA library. All clones were identical and consisted of 1159 nucleotides and 328 amino acids. Approximately 1 µl of cDNA was loaded into a 1.5% agarose gel along with a 100-nucleotide bp ladder (lane 1).

Table 2-2. Sequence identity (amino acid) between full equine GnRH receptor sequence and other mammals.

Species	**% Identity**
Ovine	90%
Bovine	91%
Porcine	91%
Human	90%
Murine	85%

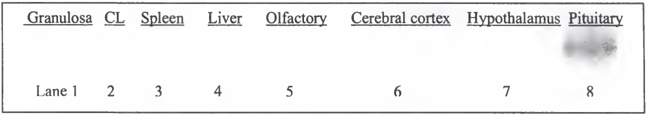

Figure 2-4. GnRH receptor mRNA expression in various equine tissues. RNA was extracted from tissues listed using the TRIzol method. Total RNA was electrophoresed through agarose gel, transferred to nitrocellulose membranes, hybridized to $^{32-}$P-labelled equine GnRH receptor cDNA, and then examined by autoradiography. Only the RNA from the anterior pituitary gland (lane 8) hybridized with the GnRH receptor cDNA.

Table 2-3. This table lists the 5 amino acid residues and their loci which are unique to the consensus equine GnRH receptor sequence compared to that of other consensus mammalian GnRH receptors. In addition, the structural change, which corresponds to each amino acid, is included as a potential source of change in receptor function.

Amino Acid	Consensus Mammalian Receptor	Charge	Equine Receptor	Charge
17	Isoleucine	Non-polar Hydrophobic	Serine	Uncharged Hydrophilic
26	Glycine	Uncharged Hydrophilic	Alanine	Non-polar Hydrophobic
61	Glutamine	Uncharged	Histidine	+ Charged
69	Lysine	+ Charged	Asparagine	Uncharged
226	Isoleucine	Non-polar	Phenylalanine	Non-polar

Discussion

The equine GnRH receptor cDNA is composed of 1159 nucleotides that encode a protein consisting of 328 amino acids (Figure 2-7). A high degree of homology (>85%) exists between the equine receptor and other mammalian GnRH receptors (Figure 2-6). The high degree of homology coincides with the conservation of key amino acid residues among mammalian GnRH receptors.

A pair of cysteine residues (Cys^{114} and Cys^{196}), believed to form a disulphide bridge between extracellular loop 1 and 2 are present in most mammalian GnRH receptors including the equine GnRH receptor (Davidson JS et al., 1994). These cysteine residues are believed to play a role in stabilizing the GnRH receptor-ligand complex via disulfide bridge formation (Keinan D and Hazum E, 1985). Additionally, several sites of N-linked glycosylation (Asn^3, Asn^{10}, Asn^{18}, Asn^{27}, and Asn^{101}) exist in the N-terminus and 1st extracellular loop of the equine GnRH receptor. Two of these residues (Asn^{18} and Asn^{101}) are most highly conserved among mammalian GnRH receptors and play a role in providing an adequate concentration of surface receptors (Davidson JS et al., 1994).

In addition to the conserved cysteine and glycosylation sites, several potential phosphorylation sites are present within the second and third intracellular loops of equine and other mammalian GnRH receptors. Site-directed mutagenesis of these highly conserved sites (Thr^{238}, Ser^{253}, and Thr^{264}) in the rat GnRH receptor indicated that these sites might play a role in the maintenance of structural integrity and expression of the GnRH receptor. However, phosphorylation of these residues does not appear to be a component of desensitization (Lin X et al., 1998b) because three of these residues are conserved in equids and the equine GnRH receptor has several additional putative phosphorylation sites within the third intracellular loop. Likewise, considering that the equine pituitary cells appear to be less sensitive to desensitization and downregulation compared to other mammals, these potential phosphorylation sites may be more important for signal transduction rather than signal attenuation.

At the cytosolic end of the third transmembrane helix and the beginning of the second cytoplasmic domain of equine and other mammalian GnRH receptors, a Ser residue replaces the Tyr residue of the 'DRY' motif (Asp-Arg-Tyr) creating a 'DRS' motif. The 'DRY" motif is highly conserved among G protein-coupled receptors and has been implicated in the coupling of receptors with the G proteins (McArdle CA et al., 1995). However, in the rat GnRH receptor, mutation of the Ser^{140} residue back to Tyr^{140} did not appear to affect G protein coupling but rather resulted in increased receptor internalization and binding affinity (Arora KK et al., 1995). The 'DRS' motif is conserved in the equine GnRH receptor suggesting that this highly conserved motif is important for GnRH receptor function independent of species.

In most G protein-coupled receptors, an aspartate residue in the second transmembrane domain is essential for agonist binding and G protein coupling. In the equine and other mammalian GnRH receptors, this residue is replaced by asparagine (Asn ^{87}). Furthermore, in the seventh transmembrane domain a highly conserved asparagine is replaced by aspartate^{318} in mammalian GnRH receptors (Cook JV et al., 1993). If both residues were mutated back to their usual locations in G protein-coupled receptors, the receptors demonstrated minimal binding to ligand or signal transduction. These data suggested that Asn^{87} may be essential for ligand binding and although Asp^{318} may not play an important role in ligand binding, it is necessary for signal transduction.

Both of these residues are conserved in the equine GnRH receptor, implicating their importance in GnRH receptor function across species.

In addition to the conservation of amino acid residues important for signal transduction, several residues have been conserved in the equine GnRH receptor that have been shown to be important for ligand binding. Work done by Sealfon and co-workers (1997) reported that His^2 of the decapeptide GnRH may interact with Lys^{121} near the top of transmembrane domain #3 whereas Arg^8 of GnRH interacts with Glu^{301} in extracellular loop #3 and the c-terminal glycinamide of GnRH interacts with Asn^{102} at the apex of transmembrane domain #2 (Sealfon SC et al., 1997). Additionally, recent work by Chauvin and co-workers (2000) demonstrated the importance of Trp^{279}, located within transmembrane domain #6, and Val^{299}, located in extracellular loop #3, for high-affinity binding to GnRH (Chauvin S et al., 2000). Site-directed mutagenesis of any or all of these residues results in a significant decline in ligand binding which does not appear to be caused by a decline in receptor number but rather a decrease in binding to cell surface receptors. All of the above mentioned residues are conserved in the equine GnRH receptor suggesting that a similar region of the equine receptor may interact with GnRH.

Unlike other G protein-coupled receptors, no carboxy-terminal cytoplasmic tail exists in the equine GnRH receptor (Figure 2-7) or in that of other mammalian GnRH receptors (Blomenrohr M et al., 1999). In those G protein-coupled receptors containing carboxy terminal tails, the tail plays an important role in rapid receptor internalization (Ferguson SS et al., 1996). Additionally, in nonmammalian GnRH receptors the intracellular carboxy terminus plays an integral part in receptor desensitization and downregulation resulting from ligand-stimulated phosphorylation of Ser and Thr residues

(Palczewski K and Benovic JL, 1991). Hence the carboxy terminus is important for mediating signal attenuation by removing surface receptors and potentially phosphorylating the receptor at key residues.

Although the equine GnRH receptor shares a high degree of homology with other species, there are several amino acid residues that are unique to the horse. These include Ser^{16}, Ala^{25}, His^{61}, Asn^{69}, and Phe^{225} (Table 2-3). In addition there are several residues that are different from those in at least 75% of other mammalian GnRH receptors including Thr^{50}, Lys^{75}, Arg^{156}, and Leu^{314}. The unique amino acid residues coincide with the N-terminus (Ser^{16} and Ala^{25}), the first intra-cellular loop (His^{61} and Asn^{69}) and the fifth transmembrane domain (Phe^{225}). It remains possible that the amino acid residues within the N-terminus which are unique to the horse, might impart distinct qualities with respect to ligand interaction. Moreover, the two unique amino acid residues within the first intracellular loop may impart variations in signal transduction regulation. Point-mutation studies are required to investigate the role these unique amino acid residues play in the equine GnRH receptor.

It quickly becomes clear from this discussion that the equine GnRH receptor has retained many if not all the residues known to be important in regulating receptor function. However, *in vivo*, the equine pituitary gland does not respond to continuous ligand exposure in the same manner as other mammalian pituitary glands. Rather than become desensitized to the constant GnRH treatment and undergo receptor downregulation, mares treated with continuous GnRH responded with continued LH output (Porter MB et al., 1997b). This is in stark contrast to the apparent downregulation noted in ewes treated with continuous GnRH, which has been reported by others (Nett TM et al., 1981).

Downregulation of the GnRH receptor has been associated with a decline in surface-bound receptors due to internalization and suppression of gene transcription and/or translation. A reduction in gene transcription would contribute to the decline in available cell-surface GnRH receptors hence promoting downregulation. Time- and dose-dependent reductions in the level of receptor mRNA were observed after treatment of αT3-1 cells with GnRH analogs (Mason DR et al., 1994). In this same study, GnRH receptor concentration at the cell surface fell by nearly 50% after 2h treatment with 10^{-8} M GnRH analog. This suggests that a component of downregulation in αT3-1 cells is due to a decline in cell-surface receptors that is not compensated for by receptor recycling. In addition to the decline in GnRH receptor mRNA levels, studies in αT3-1 cells have also indicated decreased translation to be a component of GnRH receptor downregulation. A 40% reduction in the ability of RNA to direct synthesis of functional GnRH receptors was noted in αT3-1 cells after being exposed to GnRH for 24 hours (Tsutsumi M et al., 1995). In this study, GnRH receptor mRNA levels were not altered with GnRH treatment suggesting that the downregulation of GnRH receptors in αT3-1 cells may be due in part to decreased translational efficiency.

Therefore alternative mechanisms may exist that explain the equine GnRH receptor's insensitivity to signal attenuation. These would include variations in the rate of receptor recycling, the signal transduction pathway(s), and GnRH receptor expression. The data from this study have not eliminated the possibility that the equine GnRH receptor has a subtle difference in amino acid structure, which contributes to the insensitivity to signal attenuation described *in vivo.* Although there are only 5 residues that are completely novel to the horse, based on their location, any one of them could be playing a key role in receptor function.

```
Equine  1    MANSASPEQNQNHCSASNSSIPLTQANLPTLTLSGKIRVTVTFFLFLLSTTFNASFLLKL 60
Swine   1    MANSASPEQNQNHCSAINSSILLTQGNLPTLTLSGKIRVTVTFFLFLLSTAFNASFLLKL 60
Human   1    MANSASPEQNQNHCSAINNSIPLMQGNLPTLTLSGKIRVTVTFFLFLLSATFNASFLLKL 60
Canine  3       SASPEQNQNHCSAVNNSNMLMQGNLPTLTLSGKIRVTVTFFLFLLSTIFNASFLLKL 59
Bovine  1    MANSDSPEQNENHCSAINSSIPLTPGSLPTLTLSGKIRVTVTFFLFLLSTIFNTSFLLKL 60
Ovine   1    MANGDSPDQNENHCSAINSSILLTPGSLPTLTLSGKIRVTVTFFLFLLSTIFNTSFLLKL 60
Primate 1    MANSALPEQNQNHCSVINNSIPLMQGNLPTLTLSGKIRVTVTFFLFLLSATFNASFLLKL 60
Mouse   1    MANNASLEQDPNHCSAINNSIPLIQGKLPTLTVSGKIRVTVTFFLFLLSTAFNASFLLKL 60
Rat     1    MANNASLEQDQNHCSAINNSIPLTQGKLPTLTLSGKIRVTVTFFLFLLSTAFNASFLVKL 60

Equine  61   HKWTQKKENGKKLSKMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYL 120
Swine   61   QKWTQRKEKGKKLSRMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYL 120
Human   61   QKWTQKKEKGKKLSRMKLLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYL 120
Canine  60   QKWTQKKEKGKKLSRMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYL 119
Bovine  61   QNWTQRKEKRKKLSRMKLLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYL 120
Ovine   61   QNWTQRKEKRKKLSKMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYL 120
Primate 61   QKWTQKKEKGKKLSRMKLLLKHLTLANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYL 120
Mouse   61   QKWTQKRKKGKKLSRMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYL 120
Rat     61   QRWTQKRKKGKKLSRMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGEFLCKVLSYL 120

Equine  121 KLFSMYAPAFMMVVISLDRSLAITRPLAVKSNSKLGRSMIGLAWLLSSIFAGPQLYIFRM 180
Swine   121 KLFSMYAPAFMMVVISLDRSLAITRPLAVKSNSRLGRFMIGLAWLLSSIFAGPQLYIFRM 180
Human   121 KLFSMYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVGLAWILSSVFAGPQLYIFRM 180
Canine  120 KLFSMYAPAFMMVVISLDRSLAITRPLAMKNNGKLGQSMIGLAWLLSGIFAGPQLYIFRM 179
Bovine  121 KLFSMYAPAFMMVVISLDRSLAITKPLAVKSNSKLGQFMIGLAWLLSSIFAGPQLYIFGM 180
Ovine   121 KLFSMYAPAFMMVVISLDRSLAITRPLAVKSNSKLGQFMIGLAWLLSSIFAGPQLYIFGM 180
Primate 121 KLFSMYAPAFMMVVISLDRSLAITRPLALKSSSKLGQSMVGLAWILSSVFAGPQLYIFRM 180
Mouse   121 KLFSMYAPAFMMVVISLDRSLAITQPLAVQSNSKLEQSMISLAWILSIVFAGPQLYIFRM 180
Rat     121 KLFSMYAPAFMMVVISLDRSLAVTQPLAVQSKSKLERSMTSLAWILSIVFAGPQLYIFRM 180

Equine  181 IHLADSSGQTEGFSQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLLFMLICNAKIIFTLTR 240
Swine   181 IHLADSSGQTEGFSQCVTHGSFPQWWHQAFYNFFTFSCLFIIPLLIMLICNAKIMFTLTR 240
Human   181 IHLADSSGQTKVFSQCVTHCSFSQWWHQAFYNFFTFSCLFIIPLFIMLICNAKIIFTLTR 240
Canine  180 IHLADSSGQTEGFPQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLFITLICNAKIIFTLTR 239
Bovine  181 IHLADDSGQTEGFSQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLLIMVICNAKIIFTLTR 240
Ovine   181 IHLADDSGQTEGFSQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLLIMLICNAKIIFTLTR 240
Primate 181 IHLADSSGQTKVFSQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLLIMLICNAKIIFTLTR 240
Mouse   181 IYLADGSGPTV-FSQCVTHCSFPQWWHQAFYNFFTFGCLFIIPLLIMLICNAKIIFALTR 239
Rat     181 IYLADGSGPAV-FSQCVTHCSFPQWWHEAFYNFFTFSCLFIIPLLIMLICNAKIIFALTR 239

Equine  241 VLHQDPHKLQLNQSKNNIPRARLRTLKMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRV 300
Swine   241 VLQQDPHNLQLNQSKNNIPRARLRTLKMTVAFAASFIVCWTPYYVLGIWYWFDPEMVNRV 300
Human   241 VLHQDPHELQLNQSKNNIPRARLKTLKMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRL 300
Canine  240 VLHQDPHELQLNQSKNNIPRARLRTLKMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRV 299
Bovine  241 VLHQDPHKLQLNQSKNNIPRARLRTLKMTVAFATSFTVCWTPYYVLGIWYWFDPDMVNRV 300
Ovine   241 VLHQDPHKLQLNQSKNNIPQARLRTLKMTVAFATSFTVCWTPYYVLGIWYWFDPDMVNRV 300
Primate 241 VLHQDPHKLQLNQSKNNIPRARLKTLKMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRV 300
Mouse   240 VLHQDPRKLQLNQSKNNIPRARLRTLKMTVAFATSFVVCWTPYYVLGIWYWFDPEMLNRV 299
Rat     240 VLHQDPRKLQLNQSKNNIPRARLRTLKMTVAFGTSFVICWTPYYVLGIWYWFDPEMLNRV 299

Equine  301 SDPVNHFFFLFALLNPCFDPLIYGYFSL 328
Swine   301 SDPVNHFFFLFAFLNPCFDPLIYGYFSL 328
Human   301 SDPVNHFFFLFAFLNPCFDPLIYGYFSL 328
Canine  300 SDPVNHFFFLFALLNPCFDPLIYGYFSL 327
Bovine  301 SDPVNHFFFLFAFLNPCFDPLIYGYFSL 328
Ovine   301 SDPVNHFFFLFAFLNPCFDPLIYGYFSL 328
Primate 301 SDPVNHFFFLFAFLNP             316
Mouse   300 SEPVNHFFFLFAFLNPCFDPLIYGYFSL 327
Rat     300 SEPVNHFFFLFAFLNPCFDPLIYGYFSL 327
```

Figure 2-5. Deduced amino acid sequence alignment of various mammalian GnRH receptors

```
Equine  1   MANSASPEQNQNHCSASNSSIPLTQANLPTLTLSGKIRVTVTFFLFLLSTTFNASFLLKL 60
Swine   1   ................I....L...G........................A......... 60
Human   1   ................I.N....M.G.......................A.......... 60
Canine  3      .............V.N.NM.M.G........................I......... 59
Bovine  1   ....D.....E.....I.......PGS.......................I..T...... 60
Ovine   1   ...GD..D..E.....I....L..PGS.......................I..T...... 60
Primate 1   .....L.........VI.N....M.G.......................A.......... 60
Mouse   1   ...N..L..DP.....I.N....I.GK.....V.................A......... 60
Rat     1   ...N..L..D......I.N......GK.......................A......V.. 60

Equine  61  HKWTQKKENGKKLSKMKVLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELLCKVLSYL 120
Swine   61  Q....R..K.....R....................................F........ 120
Human   61  Q.......K.....R..L.......................................... 120
Canine  60  Q.......K.....R....................................F........ 119
Bovine  61  QN...R..KR....R..L.......................................... 120
Ovine   61  QN...R..KR.................................................. 120
Primate 61  Q.......K.....R..L.................................F........ 120
Mouse   61  Q.....RKK.....R....................................F........ 120
Rat     61  QR....RKK.....R....................................F........ 120

Equine  121 KLFSMYAPAFMMVVISLDRSLAITRPLAVKSNSKLGRSMIGLAWLLSSIFAGPQLYIFRM 180
Swine   121 .................................R...F...................... 180
Human   121 ............................L.....V.Q..V....I...V........... 180
Canine  120 ............................M.N.G...Q..........G............ 179
Bovine  121 ........................K...........QF....................G. 180
Ovine   121 ....................................QF....................G. 180
Primate 121 ............................L..S....Q..V....I...V........... 180
Mouse   121 ........................Q....Q.....EQ...S...I..IV........... 180
Rat     121 ......................V.Q....Q.K...E...TS...I..IV........... 180

Equine  181 IHLADSSGQTEGFSQCVTHCSFPQWWHQAFYNFFTFSCLFIIPLLFMLICNAKIIFTLTR 240
Swine   181 ...................G.........................I........M..... 240
Human   181 ..........KV..........S.....................FI.............. 240
Canine  180 .............P..............................FIT............. 239
Bovine  181 .....D.......................................I.V............ 240
Ovine   181 .....D.......................................I.............. 240
Primate 181 ..........KV.................................I.............. 240
Mouse   181 .Y...G..P.V-........................G........I..........A... 239
Rat     181 .Y...G..PAV-...............E.................I..........A... 239

Equine  241 VLHQDPHKLQLNQSKNNIPRARLRTLKMTVAFATSFTVCWTPYYVLGIWYWFDPEMLNRV 300
Swine   241 ..Q....N.........................A..I...................V... 300
Human   241 .......E...............K...................................L 300
Canine  240 .......E.................................................... 299
Bovine  241 ......................................................D.V... 300
Ovine   241 ...................Q..................................D.V... 300
Primate 241 .......................K.................................... 300
Mouse   240 ......R.............................V....................... 299
Rat     240 ......R.........................G...VI...................... 299

Equine  301 SDPVNHFFFLFALLNPCFDPLIYGYFSL 328
Swine   301 ............F............... 328
Human   301 ............F............... 328
Canine  300 ............................ 327
Bovine  301 ............F............... 328
Ovine   301 ............F............... 328
Primate 301 ............F...             316
Mouse   300 .E..........F............... 327
Rat     300 .E..........F............... 327
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Figure 2-6. Deduced amino acid sequence of GnRH receptor. Dots correspond to homology between equids and other species while letters correspond to different residues

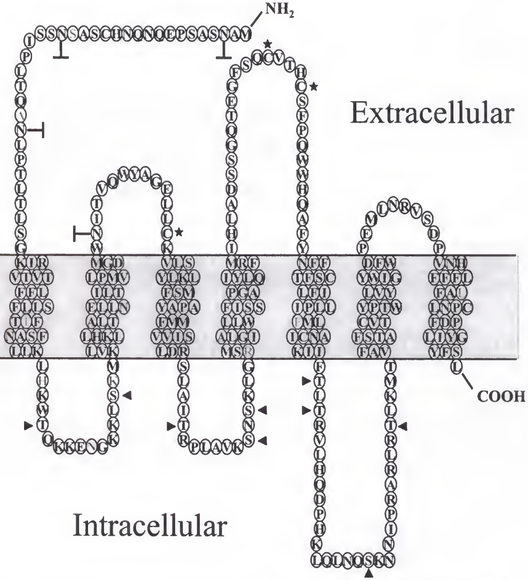


Figure2-7. Proposed seven transmembrane topography of the equine GnRH receptor. The shaded region represents the transmembrane domain. Potential N-linked glycosylation sites are marked by the symbol, " ⊥", potential intracellular phosphorylation sites are marked by solid triangles (▲), and cysteine residues are indicated by solid stars (★)

CHAPTER 3

EQUINE GNRH RECEPTOR BINDING KINETICS: MAINTENANCE OF AVAILABLE SURFACE-BOUND RECEPTORS DURING GNRH TREATMENT

Introduction

The mechanism(s) of internalization and recycling of the GnRH receptor are of special interest considering the structural distinctions between mammalian GnRH receptors and other G protein-coupled receptors. In most G protein-coupled receptors, ligand binding to receptor is followed by phosphorylation of key residues within the cytoplasmic carboxy-terminal tail of the receptor (Innamorati G et al., 1999). Moreover, internalization of the receptor-ligand complex is dependent on phosphorylation of these key residues. Once internalized, de-phosphorylation of the receptor is required for recycling of the receptor back to the plasma membrane such that incorporation of phosphate into the receptor prevents recycling and the receptor remains within the cytosol. Interestingly, a single mutation in the carboxy terminus accelerates de-phosphorylation of G protein-coupled receptors and increases the rate of receptor recycling (Innamorati G et al., 1999). However, the mammalian GnRH receptor lacks a cytoplasmic carboxy-terminal tail and potential sites for phosphorylation (serine and threonine residues) are absent within the second and third intracellular loops of the receptor. Vrecl and co-workers (1998) studied agonist-induced endocytosis and recycling of the rat GnRH receptor (Vrecl M et al., 1998). Their study demonstrated that, despite lacking an intracellular carboxy-terminal tail, the rat GnRH receptor underwent GnRH-induced internalization. More recently, Heding and co-workers (2000) compared GnRH receptor internalization in the rat versus the catfish (Heding A et al., 2000). Interestingly, the catfish receptor internalized almost twice as fast as the rat GnRH. This difference in rate of internalization was attributed to the cytoplasmic carboxy-terminal tail located in the catfish GnRH receptor yet absent from the rat receptor. Similar results were reported by Pawson and co-workers (1998), comparing the rate of internalization between the human and chicken GnRH receptors. As in the study between the rat (mammalian) and catfish (non-mammalian) GnRH receptor, the mammalian GnRH receptor (human) internalized at a significantly slower rate than that of the non-mammalian (chicken) due to the lack of a cytoplasmic C-terminal tail.

Unfortunately, little is known regarding the rate of GnRH receptor internalization and recycling in other species. However, the preservation of key amino acid residues among most mammalian GnRH receptors increases the likelihood of similar internalization and recycling kinetics. One such amino acid residue is Alanine^{261} located in the third intracellular loop of the GnRH receptor. When Ala^{261} was mutated to Lys^{261} there was a significant decrease in receptor internalization (Myburgh DB et al., 1998a). Furthermore, mutation of Ala^{261} resulted in complete uncoupling of the receptor from the associated G proteins. This residue is conserved in swine, human, canine, bovine, ovine, primate, muridae, and equine GnRH receptor.

As described in Chapter 2, the equine GnRH receptor retains greater than 85% identity with the GnRH receptor from the above-mentioned species. However, *in vivo* data regarding the gonadotropic response to continuous GnRH treatment in ewes versus pony mares suggests variations in the mechanism(s) of signal transduction in pony mares (Porter MB et al., 1997b). Although the ovine and pony GnRH receptor are 90%

homologous in amino acid structure, sheep responded to continuous infusion of GnRH by an initial increase in LH secretion followed by a sustained decline when exposed to continued GnRH exposure. In contrast, pony mares infused with continuous GnRH responded with sustained LH secretion above basal levels throughout the infusion period (15 hr). Nett and co-workers (1981) first described the response to continuous GnRH exposure in sheep. These authors observed maximum serum concentrations of LH following 2 hours of GnRH infusion, at which time levels of LH began to decline and returned to baseline between 8 and 20 hours, as GnRH infusions continued. The decline in LH response was attributed to down-regulation of the number of receptors for GnRH by 12 hours (Nett TM et al., 1981). Similar observations were made in primates (Knobil E, 1981), pigs (Chakraborty PK et al., 1973), and rats (Koiter TR et al., 1979).

Therefore, although the equine GnRH receptor is quite similar in structure to that of other mammals, it is unique in response to continuous GnRH exposure. As mentioned previously, the unique qualities of the pony's GnRH receptor may involve variations in GnRH receptor structure, variations in GnRH receptor kinetics, or distinctions in the method of signal attenuation. Potential variations in the receptor kinetics might include differences in rates of internalization and/or accelerated rates of recycling such that equine gonadotropes are capable of responding to continuous GnRH by maintaining adequate concentrations of receptors on the cell surface. Furthermore, equine GnRH receptor affinity for GnRH and/or receptor number may differ from that in other mammals. Therefore, the following experiments were designed to examine the equine GnRH receptor and its binding kinetics. Determination of equilibrium constants (K_d &

K_a) for the equine GnRH receptor as well as internalization and recycling constants (k_e & V_r) will allow comparison between the GnRH receptor in horses versus other mammals.

Materials and Methods

Pituitary cell dispersion protocol

Pituitary glands were collected from horses within 15 minutes after euthanasia. The anterior pituitary was dissected free of the posterior pituitary and placed in sterile transport medium at 4° C (25mM Hepes, 5mM KCl, 137mM NaCl, 10mM Glucose, 0.4% BSA and 1% antibiotic-antimycotic, pH = 7.4). After incubation at 4° C for 30-60 minutes, pituitary tissue was placed into a petri dish and bisected into 5x5 mm (approximate) segments. The tissue fragments were then washed three times in cold transport medium and placed on ice for transport to the laboratory. In preparation for enzymatic dispersion, pituitary tissue fragments were further bisected into 1X1 mm segments and then washed 3 times in cold transport medium. Tissue fragments were placed in a trypsinizing flask (150 ml) along with enzymatic dispersion solution (transport media (50ml), 0.25 % Collagenase Type II (Sigma C-6885), and 0.002 % DNAse (Sigma D-4263) at 37° C). The flask was placed on a stir plate within an incubator set at 37° C for 5 minutes, after which the dispersion solution was removed (minus tissue fragments) and centrifuged to remove non-viable pituitary cells and red blood cells (RBC). Subsequently, the dispersion solution was added back to tissue fragments and allowed to incubate at 37° C for 10 minutes on a stir plate. After 10 minutes of stirring, the dispersion solution was removed (minus tissue fragments) and centrifuged to collect dispersed pituitary cells. The dispersion solution (minus dispersed pituitary cells) was added back to the tissue fragments for another 10 minutes. This was repeated 4-6 times or until all tissue fragments were completely dispersed. The recovered pituitary cells were washed 3 times in transport media at 37° C to remove RBC's and then passed through a 20 µM nylon filter (Fischer) to eliminate cellular debris. After dispersion was complete, total cell count and viability were determined using a hemocytometer and 0.04% trypan blue. The pituitary cell recovery averaged 20-50 million cells per pituitary gland.

Prior to internalization studies, equine pituitary cells were maintained in primary cell culture for 48 hours. Cells were cultured in non-adherent petri dishes containing DMEM media (Gibco DMEM pack™ (w/ Glucose, L-Glutamine, and Sodium Pyruvate) plus 25 mM Hepes, 0.25% BSA, 400 mg/L $CaCl_2$, 3.7 gm/L $NaHCO_3$, 10% heat-inactivated calf serum, and 1% antibiotic-antimycotic) under humidified conditions (37° C and 5% CO_2). Additionally, cell culture media was spiked with 10^{-10} molar GnRH (Sigma L-7134) for maintenance of GnRH receptor expression.

GnRH Iodination

Iodination of D-Alanine GnRH was performed using the protocol provided by Dr. Terry Nett (Animal Reproductive Biology Group, Colorado State University, Fort Collins CO). The protocol has been published by Dr. Nett and others and is listed in its entirety in Appendices A of this document.

Pituitary Membrane Preparation

The protocol for preparing the pituitary plasma membrane preparations was adopted from previous publications (Nett TM et al., 1981). The procedure is listed in its entirety in Appendices B of this document.

GnRH Receptor Internalization Studies

The procedure for the receptor internalization experiments was derived from the protocol published by Nett and co-workers with some modification. In brief, pituitary cells were recovered from static culture and washed (1X) in DMEM (serum-free) at 4° C. After washing, approximately 1 million cells were suspended in either 500 µl (tubes A: total binding) or 450 µl (tubes B: non-specific binding) of cold DMEM (serum-free). Internalization experiments were performed in 1.5 ml eppendorf tubes. First, 50 µl of excess (1 µg) non-radiolabelled D-Alanine GnRH (Sigma L-4513) was added to all B tubes. Secondly, 100 µl (approximately 2×10^5 cpms) of radiolabelled D-Alanine GnRH was added to all tubes (A&B). Lastly, the cell suspensions were added to all tubes (A&B). The experiments were performed in triplicate (3 A tubes and 3 B tubes per time period). After the addition of all reagents, all tubes were placed in ice and maintained at 4° C for 4 hours at which point equilibrium (rate of dissociation equaled the rate of association) was established.

Internalization of the GnRH receptor was initiated by placing the tubes in a 37° C water bath and internalization was terminated at various time points depending on experiment. In experiment I, internalization was halted at time 0, 20 min, 40 min, and 60 min. In experiment II, internalization was halted at time 0, 30 min, 60 min, 120 min, and 180 min. Termination of internalization required placing cells in an ice bath followed by brief (30-60 seconds) high speed centrifugation (4° C). The radioactive supernatant was discarded and 1 ml of cold acid solution (150mM NaCl, 50mM Acetic acid, pH 2.8) was added to the pelleted cells. The cells were gently mixed with cold acid solution and incubated at 4° C for 12 minutes. Exposure of cells to cold acid solution caused dissociation of surface bound radioligand. After 12 minutes, cells were briefly (30-60 seconds) centrifuged (4° C) at high speed. The supernatant was recovered into 12X75 polypropylene tubes, which represented surface bound radio-ligand. The bottom of the eppendorf tubes, containing the pelleted cells, was cut free from the tube and placed in 12X75 plastic tubes. The radioactivity within the supernatant and pelleted cells was counted with a gamma counter and converted to molar concentrations of radioligand. Calculation of surface bound receptor was determined by adjusting the total binding (tubes A), measured in the supernatant following acid wash, by non-specific binding (tubes B). Like wise, the estimation of internalized receptor was based on the radioactivity detected in the pelleted fractions, after adjusting for non-specific binding. The molar concentrations of radioligand measured on the surface and inside the cell were plotted over time. Analysis of variance was performed on the triplicate values measured at each time point to determine the main effect of time on radioligand concentration within the cells and on the surface. Additionally, regression analysis was performed on the time trends to characterize the order of the regression curves generated for experiment I and II.

Electron Microscopy

In addition to the GnRH receptor internalization experiments, equine pituitary cells were harvested from pony mares, during late estrus, as previously described (pituitary cell dispersion protocol). Pituitary cells were maintained in static culture for 48 hours prior to initiation of GnRH treatment. After 48 hours, cells were recovered from petri dishes, washed in culture media (1x), and resuspended in culture media containing 10^{-8} M GnRH. Exposure to GnRH was halted at time 0, 10 min, 20 min, 40 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, and 7 hr. At each time point, pituitary cells were recovered from petri dishes (in duplicate) and briefly centrifuged. The pelleted cells were fixed in 4% para-formaldehyde and the Electron Microscopy Core Laboratory at the University of Florida performed the remainder of tissue preparation, including electron microscopy photography. The sections of pituitary cells were exposed to colloid gold-labeled antibodies directed against GnRH (rabbit polyclonal IgG, LH-RH IHC 960647, Peninsula Laboratories, Inc., San Carlos, CA) and GnRH receptor (goat polyclonal IgG, GnRHR sc-8681, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This allowed for simultaneous visualization of ligand (GnRH) and GnRH receptor.

Saturation Binding Experiments

Saturation binding experiments were performed using equine pituitary membrane preparations and dispersed equine pituitary cells to estimate the dissociation constant (K_d) and receptor number (Bmax). The saturation binding experiments consisted of incubating equal concentrations of pituitary membranes or pituitary cells with increasing amounts of radiolabelled GnRH. Subsequently, Bmax and equilibrium dissociation constants (K_d) were determined via linear regression (Scatchard plot) and non-linear regression methodologies. Non-specific binding (NSB) was determined at all concentrations of radiolabelled D-Alanine GnRH added by including samples with 100-fold excess of unlabelled D-Alanine GnRH. Total radioactivity (CPMs) was adjusted for NSB and then converted to picomolar (pM) concentrations based on the estimated specific activity (2754 CPM/picogram) of radiolabelled D-Alanine GnRH. For the non-linear regression analysis, bound GnRH concentrations (pM) were plotted on the Y-axis and free GnRH concentrations (pM) were plotted on the x-axis. Using computer software (Prism), the data were fit to a saturation binding curve equation (one site binding) and the K_d and Bmax were estimated. For the linear regression Scatchard plot analysis was performed and the ratio of bound radioligand over free radioligand was plotted on the y-axis versus bound radioligand on the x-axis. The slope of the line equaled $-K_a$ or K_d^{-1} and the X-intercept (Y=0) was an estimate of the surface receptor density (Bmax). The values for the dissociation constant (K_d) and receptor density (Bmax) were compared between linear and non-linear regression analysis plus the values obtained were compared to those previously published in the horse and other species.

Derivation of Endocytotic Rate Constant (k_e) and Dissociation Rate Constants (k_d)

The rate of receptor endocytosis (k_e) was calculated for internalization experiments I and II by plotting the ratio of the internalized radioligand to surface-bound radioligand on the y-axis versus time on the x-axis. Based on the change in the ratio of internalized versus surface bound radioligand over time, the slope of the plot is considered an estimate of the rate of receptor endocytosis (k_e) (Wiley HS and Cunningham DD, 1982). Regression analysis was also performed on the curves generated for each experiment to determine the order of the curves. In addition, the dissociation rate constants (k_d) were calculated for both internalization experiments. The k_d represents the rate at which the ligand becomes dissociated from the receptor and is derived by plotting the natural log of the ratio of surface bound ligand over time versus surface bound ligand at time zero.

Results

Saturation Binding Experiments

Non-linear regression analysis of the saturation data estimated the dissociation constant (K_d) to equal 2.5×10^{-9} M (Figure 3-2) and 1.11×10^{-9} M (Figure 3-4) for the membrane preparations and pituitary cells, respectively. In addition, the estimates of GnRH receptor concentration (Bmax) were 1.5×10^{-10} M per mg of protein in equine pituitary membrane preparations (Figure 3-2) and 2.7×10^{-11} M per five hundred thousand pituitary cells (Figure 3-4). Linear transformation of binding data via Scatchard Plot analysis reported K_d values equal to 5.7×10^{-8} M for membrane preparations (Figure

3-1) and 1.5 x 10^{-9} M for dispersed pituitary cells (Figure 3-3). The Bmax estimates from the Scatchard plot analysis were 3.2 X 10^{-10} M per mg of protein in equine pituitary membrane preparations (Figure 3-1) and 3.1 x 10^{-11} M per five hundred thousand pituitary cells (Figure 3-3). Comparison of the data obtained from linear versus non-linear regression analysis indicated that either method was adequate for estimating receptor concentration (Bmax), however the dissociation constant calculated from the Scatchard plots was 10-fold higher in the membrane preparations (Table 3-1). A previous publication reported a slightly lower K_d (2.1 x 10^{-8} M) for equine pituitary membrane preparations and lower concentration of GnRH receptor number (0.13 to 2.6 x 10^{-12} mol/g of tissue) in the membrane preparations (Hart et al., 1984).

GnRH Receptor Internalization Experiments (I & II)

Internalization experiment II and I were performed using dispersed equine pituitary cells. In experiment I, internalization of receptor was halted at time 0, 20, 40, and 60 minutes. There was a significant ($p<0.001$) effect of time which was due to the initial decline in surface binding from time 0 to 20 minutes, which was associated with an increase in internalized ligand-receptor complex (Figure 3-5), followed by a significant ($p<0.001$) increase in surface binding at 40 and 60 minutes. The concentration of surface-bound radioligand at 60 minutes (28.65 ±1.3 pMol) was approximately 1.5-fold greater than surface binding at time zero (21.6±1.2 pMol) (Figure 3-5). The internalized radio-ligand reached steady state within 30 minutes and was approximately 30% of total cell - associated ligand (Figure 3-5). In internalization experiment II, internalization of ligand-receptor complex was halted at time 0, 30, 60, 120, and 180 minutes. As in experiment I, there was a significant ($p<0.001$) effect of time on the concentration of surface-bound and internalized radioligand. There was an initial decline in surface

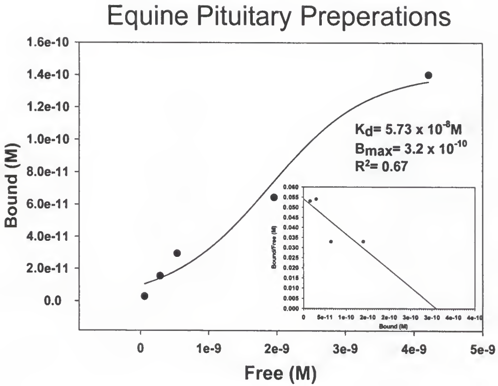


Figure 3-1 Scatchard plot analysis for the equilibrium binding of equine GnRH receptor in equine pituitary membrane preparations. Equal quantities (100 ug) of membrane preparations were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for linear regression. The K_d and Bmax were estimated based on the slope of the Scatchard plot and the x intercept.

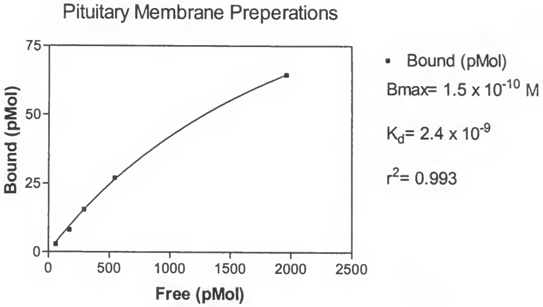


Figure 3-2 Non-linear regression analysis for the equilibrium binding of equine GnRH receptor in equine pituitary membrane preparations. Equal quantities (100 ug) of membrane preparations were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for non-linear regression. The data were entered into a one site binding equation also known as a saturation binding curve and the K_d and Bmax were determined by calculating the slope and the estimated Bmax.

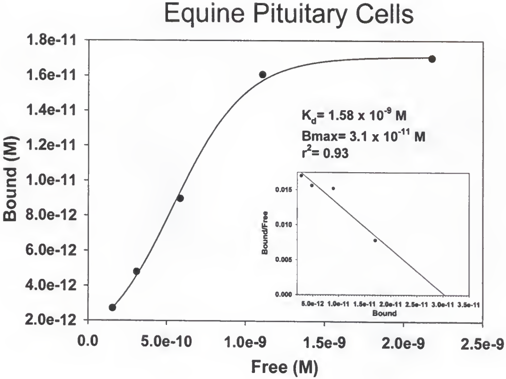
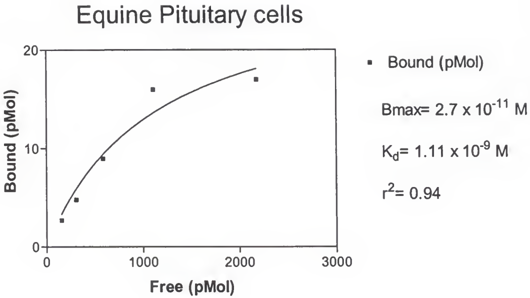


Figure 3-3 Scatchard plot analysis for the equilibrium binding of equine GnRH receptor in dispersed equine pituitary cells. Equal quantities (5 x 10^5) of equine pituitary cells were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for linear regression. The K_d and Bmax were estimated based on the slope of the Scatchard plot and the x intercept.



Equine Pituitary cells

Bmax= 2.7×10^{-11} M

K_d= 1.11×10^{-9} M

r^2= 0.94

Figure 3-4 Non-linear regression analysis for the equilibrium binding of equine GnRH receptor in dispersed equine pituitary cells. Equal quantities (5×10^5) of pituitary cells were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for non-linear regression. The data were entered into a one site binding equation also known as a saturation binding curve and the K_d and Bmax were determined by calculating the slope and estimating the X intercept.

binding followed by a significant ($p<0.0.001$) increase in surface binding at time 60, 120, and 180 minutes (Figure 3-6). In addition, radioligand concentration measured within the cells significantly increased at each time point beginning at 30 minutes (Figure 3-6). The concentration of surface-bound radioligand at 180 minutes (19.8±0.9 pMol) was approximately 2-fold greater than surface binding at time 0 (11.5±0.9 pMol) (Figure 3-6).

In addition to quantifying changes in surface-bound and internalized radioligand, the rate of endocytosis (k_e), and the dissociating rate constants (k_d) were calculated (Table 3-2). Derivation of the endocytotic rate constant (ke) was based on the concept that over time the concentration of internalized radioligand will increase as the concentration of surface-bound radioligand decreases due to internalization of receptor-ligand complexes. A plot of such a curve is predicted to yield a 1st order curve with a positive slope. Regression analysis of the curves generated when the ratio of internal radioligand to surface-bound radioligand were plotted over time, indicated that the data from both internalization experiments were best described by 3rd order curves rather than a 1st order curve. In deed, the data from both experiments appeared to have a positive slope in the early sampling periods (0.04 min^{-1} and 0.01 min^{-1}, exp I and II respectively) which subsequently declined and approached a slope of zero. The decline in slope over time was due to increasing concentration of surface-bound radioligand which lowered the ratio of internalized radioligand to surface-bound radioligand and hence, decreased the k_e estimates. In experiment I, the k_e calculated over the entire sampling period of 60 minutes (0.007 min^{-1}) was one order of magnitude slower than that published for the rat GnRH receptor (0.012 min^{-1}). Furthermore, the k_e estimate for experiment II (0.001 min^{1}) which

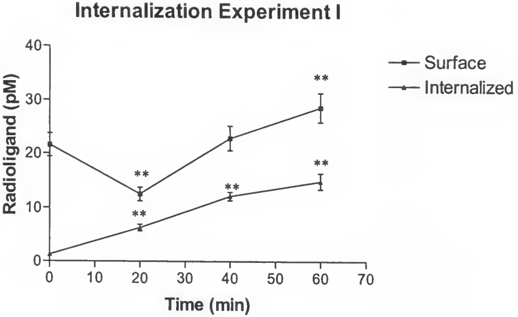


Figure 3-5 Surface binding and internalization pattern of equine GRH receptor in dispersed equine pituitary cells in experiment I. Cultures of pituitary cells (1-3 x 10^{-6}) were incubated at 4° C for 4 hours with equal concentrations of ^{125} D-Alanine GnRH. Cells were then placed at 37° C for different time periods (0, 20, 40, and 60 min) to initiate receptor-ligand complex internalization. Surface bound radioligand was removed with an acidic solution followed by centrifugation and the internalized radioligand remained behind in the cell pellet. Specific binding was determined by adjusting the total binding for non-specific binding and the binding in counts per minute was converted to molar concentrations of radioligand. Each time point represents the mean of triplicates. Analysis of variance indicated that there was a significant ($p<0.001$) effect of time which was due to the initial decline in surface binding from time 0 to 20 minutes, which was associated with an increase in internalized ligand-receptor complex, followed by a significant ($p<0.001$) increase in surface binding at 60 minutes. The concentration of surface-bound radioligand at 60 minutes (28.65 ±1.3 pMol) was approximately 1.5-fold greater than surface binding at time zero (21.6±1.2 pMol). Double "**" represent mean values which were highly ($p<0.001$) significantly different from time zero means whereas single "*" represent p values <0.01.

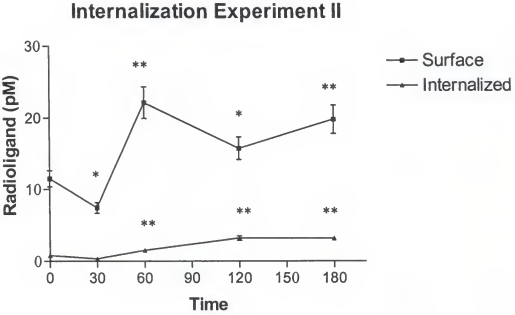


Figure 3-6 Surface binding and internalization pattern of equine GRH receptor in dispersed equine pituitary cells in experiment II. Cultures of pituitary cells ($1\text{-}3 \times 10^{-6}$) were incubated at 4° C for 4 hours with equal concentrations of ^{125} D-Alanine GnRH. Cells were then placed at 37° C for different time periods (0, 30, 60, 120, and 180 min) to initiate receptor-ligand complex internalization. Surface bound radioligand was removed with an acidic solution followed by centrifugation and the internalized radioligand remained behind in the cell pellet. Specific binding was determined by adjusting the total binding for non-specific binding and the binding in counts per minute was converted to molar concentrations of radioligand. Each time point represents the mean of triplicates. Analysis of variance indicated that there was a significant ($p<0.001$) effect of time which was due to the initial decline in surface binding from time 0 to 30 minutes, which was associated with an increase in internalized ligand-receptor complex, followed by a significant ($p<0.001$) increase in surface binding at 60, 120, and 180 minutes. The concentration of surface-bound radioligand at 180 minutes (19.80 ±.9 pMol) was approximately 2-fold greater than surface binding at time zero (11.5±.9 pMol). Double "**" represent mean values which were highly ($p<0.001$) significantly different from time zero means whereas single "*" represent p values <0.01.

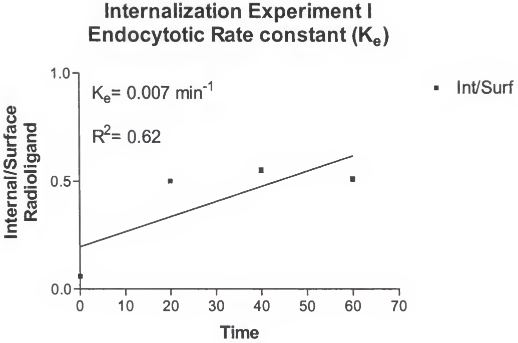


Figure 3-7 Endocytotic rate constant (k_e) of equine GnRH receptor in dispersed equine pituitary cells in experiment I. Cultures of pituitary cells (1-3 x 10^{-6}) were incubated at 4° C for 4 hours with equal concentrations of ^{125} D-Alanine GnRH. Cells were then placed at 37° C for different time periods (0, 20, 40, and 60 min) to initiate receptor-ligand complex internalization. Surface bound radioligand was removed with an acidic solution followed by centrifugation and the internalized radioligand remained behind in the cell pellet. Specific binding was determined by adjusting the total binding for non-specific binding and the binding in counts per minute was converted to molar concentrations of radioligand. The k_e was determined by plotting the ratio of internalized radioligand to surface-bound radioligand over time and the slope of the curve generated corresponds to the endocytotic rate constant.

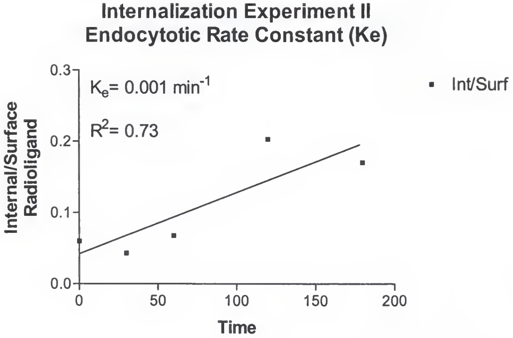


Figure 3-8 Endocytotic rate constant (k_e) of equine GnRH receptor in dispersed equine pituitary cells in experiment II. Cultures of pituitary cells (1-3 x 10^{-6}) were incubated at 4° C for 4 hours with equal concentrations of ^{125} D-Alanine GnRH. Cells were then placed at 37° C for different time periods (0, 30, 60, 120, and 180 min) to initiate receptor-ligand complex internalization. Surface bound radioligand was removed with an acidic solution followed by centrifugation and the internalized radioligand remained behind in the cell pellet. Specific binding was determined by adjusting the total binding for non-specific binding and the binding in counts per minute was converted to molar concentrations of radioligand. The k_e was determined by plotting the ratio of internalized radioligand to surface-bound radioligand over time and the slope of the curve generated corresponds to the endocytotic rate constant.

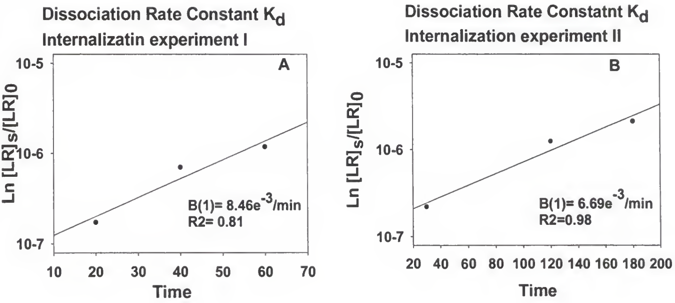


Figure 3-9 Dissociation rate constant (k_d) of equine GnRH receptor in dispersed equine pituitary cells in experiment I (A) and II (B). Cultures of 1-3 x 10^{-6} cells were incubated at 4° C for 4 hours with equal concentrations of ^{125} D-Alanine GnRH. Cells were then placed at 37° C for different time periods to initiate receptor-ligand complex internalization. Surface bound radioligand was removed with an acidic solution followed by centrifugation and the internalized radioligand remained behind in the cell pellet. Specific binding was determined by adjusting the total binding for non-specific binding and the binding in counts per minute was converted to molar concentrations of radioligand. The k_d was determined by plotting the natural log of the ratio of surface-bound radioligand at each time point versus the surface-bound radioligand at time 0. The slope of the curve generated corresponds to the dissociation rate constant.

extended the time period from 60 to 180 minutes, indicated a further decline in the rate of receptor internalization (Figure 3-8).

Electron Microscopy

Photographic images were taken of pituitary cells that had been fixed at various time points from the onset of GnRH treatment. Quantitative analysis was not possible, however visual inspection of the photographs indicated that at time zero GnRH receptors were detected throughout the surface of the pituitary gonadotropes. Shortly after beginning GnRH treatment, GnRH and GnRH receptors were detected in association with each other (Figure 3-11). Ten minutes later, the associated complex was detected within the cell, indicating internalization of GnRH and GnRH receptors (Figure 3-12). Within 40 (Figure 3-13) and 60 minutes (Figure 3-14) after initiating GnRH treatment, GnRH receptors devoid of GnRH were detected at the surface and GnRH devoid of GnRH receptors was maintained within the cell. This pattern was noted up to 7 hours after initiating GnRH treatment (Figure 3-15).

Discussion

Previous work in our laboratory has suggested that equine gonadotropes are resistant to downregulation during continuous GnRH treatment compared to other mammals (Porter MB et al., 1997b). Furthermore, injections of slow release implants, containing high concentrations of a potent GnRH analog, resulted in a 75% ovulation rate in anestrous mares (Allen WR et al., 1987). The physiological mechanisms for these phenomena have not been described. However, several characteristics of the equine estrous cycle may have prompted mares to develop signal transduction mechanisms that would be able to maintain gonadotropin responsiveness during periods of continuous GnRH secretion. Unlike other species, the mare has a prolonged (5-7 days) period of

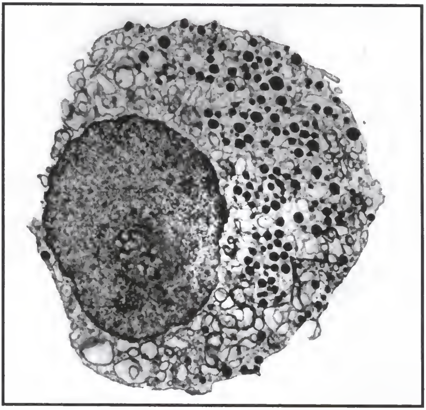


Figure 3-10 Electron micrograph of equine gonadotrope. Equine pituitary glands were enzymatically dispersed and cells were incubated in static culture for 48 hours. There after, cells were treated with GnRH and fixed in 4% paraformaldehyde at various time points (0, 10 min, 20 min, 40 min, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, and 7 hr). Following fixation, pituitary cells were sectioned and exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit) and GnRH receptor (anti-goat). Examination of post-ligand binding behavior was performed by simultaneous visualization of GnRH and GnRH receptor

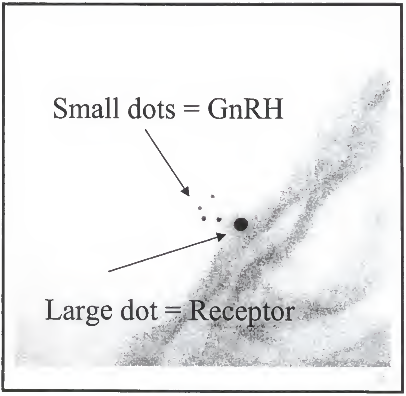


Figure 3-11 Magnification of electron micrograph containing an equine gonadotrope which has been exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit, small dots) and GnRH receptor (anti-goat, large dots). Gonadotrope was fixed after treating with GnRH demonstrating the association of several GnRH molecules with a single GnRH receptor.

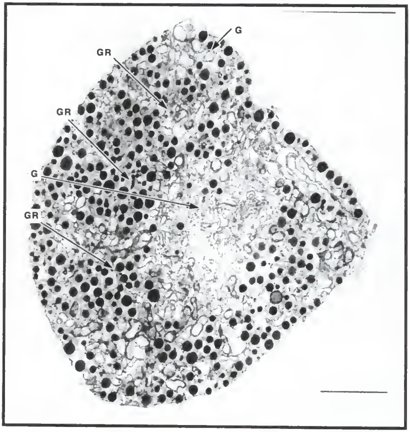


Figure 3-12 Electron micrograph of equine gonadotrope 10 minutes after initial exposure to GnRH in culture. Following fixation, pituitary cells were sectioned and exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit) and GnRH receptor (anti-goat). Examination of post-ligand binding behavior was performed by simultaneous visualization of GnRH and GnRH receptor. The letter "G" correspond to localization of free ligand (GnRH) whereas the letters "GR" correspond to localization of ligand plus GnRH receptor. After 10 minutes of GnRH treatment, this micrograph suggests that internalization of receptor-ligand complex had begun and free ligand was localized intracellularly.

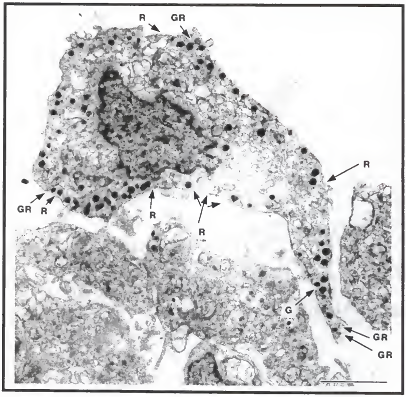


Figure 3-13 Electron micrograph of equine gonadotrope 40 minutes after initial exposure to GnRH in culture. Following fixation, pituitary cells were sectioned and exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit) and GnRH receptor (anti-goat). Examination of post-ligand binding behavior was performed by simultaneous visualization of GnRH and GnRH receptor. The letter "G" correspond to localization of free ligand (GnRH), the letter "R" corresponds to GnRH receptor and the letters "GR" correspond to localization of ligand plus GnRH receptor. After 40 minutes of GnRH treatment, this micrograph suggests that unbound GnRH receptor was present at the cell surface suggesting recycling of receptor and/or insertion of new receptor into the cells surface.

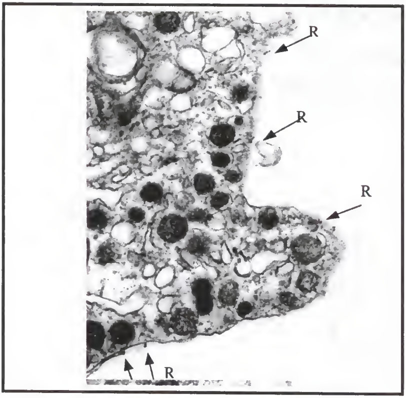


Figure 3-14 Electron micrograph of equine gonadotrope 60 minutes after initial exposure to GnRH in culture. Following fixation, pituitary cells were sectioned and exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit) and GnRH receptor (anti-goat). Examination of post-ligand binding behavior was performed by simultaneous visualization of GnRH and GnRH receptor. The letter "G" correspond to localization of free ligand (GnRH), the letter "R" corresponds to GnRH receptor and the letters "GR" correspond to localization of ligand plus GnRH receptor. After 60 minutes of GnRH treatment, this micrograph suggests that recycling and/or insertion of new receptor into the cell surface continues and may be providing new biding sites for ligand remaining in the culture media.

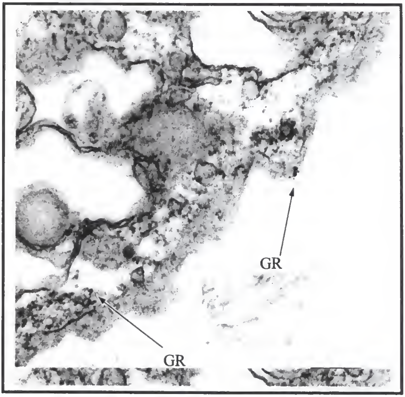


Figure 3-15 Electron micrograph of equine gonadotrope 7 hours after initial exposure to GnRH in culture. Following fixation, pituitary cells were sectioned and exposed to colloid gold-labeled antibodies directed against GnRH (anti-rabbit) and GnRH receptor (anti-goat). Examination of post-ligand binding behavior was performed by simultaneous visualization of GnRH and GnRH receptor. The letter "G" correspond to localization of free ligand (GnRH), the letter "R" corresponds to GnRH receptor and the letters "GR" correspond to localization of ligand plus GnRH receptor. After 7 hours of GnRH treatment, this micrograph suggests that a small percentage of receptor-ligand complexes have remained at the cell surface without being internalized.

estrus, during which GnRH and LH are gradually increasing. Moreover, the pre-ovulatory surge of GnRH in the mare is better described as a *gradual rise* rather than a *surge*, peaking approximately 12 to 24 hours after ovulation. In addition to the prolonged estrus period, endogenous GnRH secretion in mares has been described as irregularly episodic and continuous during estrus (Grubaugh WR and Sharp DC, 1987) (Alexander SL and Irvine CHG, 1987). GnRH secretion has also been described as continuous throughout the estrous cycle with intervening secretory events (Alexander SL and Irvine CHG, 1987). These studies suggest that GnRH secretion in mares during estrus is continuous. Thus a mechanism must exist within equine gonadotropes to maintain responsiveness to constant GnRH secretion.

Other than these *in vivo* data, little is known concerning the equine GnRH receptor. Cloning and sequencing of the receptor (Chapter 2) indicated that a high degree (>85%) of identity exists between the equine GnRH receptor and that of other mammals. Although there are several amino acid residues that are unique in the equine GnRH receptor, the studies have not been performed to determine if those variations in amino acid sequence contribute to the mare's insensitivity to GnRH receptor downregulation. However, due to the high degree of homology among GnRH receptors, the likelihood that the variations in amino acid sequence described in equine GnRH receptors is the physiological explanation for the unique *in vivo* observations is minimal. A more likely explanation for the mare's insensitivity to gonadotropic downregulation may involve mechanisms regulating the rate of receptor internalization and recycling. Therefore, this study proposed to describe the equine GnRH receptor kinetics.

Table 3-1

Binding Kinetic	Membrane Preparations	Dispersed Pituitary cells
K_D Scatchard Plot	5.7×10^{-8} M	1.5×10^{-9} M
K_D Non-linear Plot	2.5×10^{-9} M	1.1×10^{-9} M
Bmax Scatchard plot	3.2×10^{-10} M	3.1×10^{-11} M
Bmax Non-linear plot	1.5×10^{-10} M	2.7×10^{-11} M

Table 3-2

Rate Constant	Experiment I	Experiment II
k_d	8.48×10^{-3}/ min	6.69×10^{-3}/ min
k_e	0.007/ min	0.001/ min

The K_d and GnRH receptor number for equine pituitary membrane preparations (Table 3-1) were compared to those published by Hart et al., (1984) in horse mares. In that study, GnRH receptor number in pituitary membrane preparations from horse mares was compared during 4 different times of year (Hart PJ et al., 1984). The number of GnRH receptors in pituitaries ranged from 0.13 to 2.61 x 10^{-12} mol/g of tissue and the dissociation equilibrium constant (K_d) equaled 2.10 x 10^{-8} M. To date, these data remain the only published data characterizing the equine GnRH receptor. The estimates of GnRH receptor concentration (Bmax) determined by linear and non-linear regressions in our study were one order of magnitude greater compared to that previously published (Table 3-1). Additionally, the estimates for the equilibrium dissociation constants (K_d) were one order of magnitude smaller compared to that published by Hart and co-workers suggesting greater affinity. These data suggest some variation in concentration of GnRH receptor number and affinity which may be dependent on endocrine status and/or time of year. In addition the K_D and GnRH receptor number determined for equine GnRH receptors in the pituitary membrane preparations were similar in order of magnitude to published values for bovine (Cermark DL et al., 1983) and ovine GnRH receptors (Crowder ME et al., 1986).

Data from internalization experiment I and II suggest that differences may exist in rate of receptor recycling and internalization between horses and other species. In both experiments, surface binding increased rather than decreased during exposure to GnRH. This is contrary to data published in other species that described a decline in GnRH receptor number due to internalization, during exposure to GnRH. Heding et al., (1998) reported a 25% decline in surface receptor within 60 minutes after initiating GnRH treatment (Heding A et al., 1998). These studies were performed in HEK 293 cells stably transfected with the rat GnRH receptor. Furthermore, these authors reported an Endocytosis rate constant (k_e) equal to 0.012 ± .001 min^{-1} and a Recycling rate constant (k_r) equal to 0.033 ± .003 min^{-1}. In a similar study, cell surface versus internalized radiolabelled GnRH was compared in dispersed rat pituitary cells treated with GnRH (Cassina MP et al., 1997). Within 60 minutes after beginning GnRH treatment, cell surface radiolabelled GnRH was reduced by 60% whereas internalized radioligand was increased by 20%. Both cell surface and internalized GnRH reached steady state by 60 minutes of GnRH exposure. Recent data from internalization experiments in sheep pituitary cells reported a decline in surface binding and an increase in internalized receptor within 1 hr of the onset of internalization (Hashizume T et al., 2001). At 60 minutes post onset of internalization, surface-bound radioligand reached a steady state, which was 40% less than initial value. Furthermore, internalized radioligand reached steady state after 60 minutes, which was 30% of total cell-associated ligand. Results from this study suggest that the equine GnRH receptor responds to GnRH treatment by increasing the availability of surface receptors. The significant increase in surface-bound radioligand over time noted in experiments I and II suggests that new GnRH receptors are being inserted into the plasma membrane in response to continuous GnRH exposure. It remains to be determined if the increased surface binding represents the insertion of recently translated protein or mere receptor recycling.

The derivation of the endocytotic rate constant (k_e) was complicated by the increase in surface binding over time. Typically, estimation of the k_e is dependent on a continued decline in surface-bound radioligand due to internalization and simultaneous increase in internalized radioligand concentration. Regression analysis performed on the curves representing the change in internalized radioligand to surface-bound radioligand over time indicated that initially, the receptor was internalized at a rate with the same order of magnitude from that published in rats. However, as time progressed the k_e slowed due to an increase in concentration of surface-bound radioligand. It remains to be determined whether the apparent slowed rate of endocytosis corresponds to a true decline in receptor internalization or if it is due to the insertion of new receptor into the plasma membrane. Regardless of the cause, the increase in available receptors at the cell surface remains a behavior of the GnRH receptor as of yet not described. Furthermore, it provides an explanation for the ability of equids to maintain gonadotropic response during continuous GnRH stimulation.

In addition to the endocytotic rate constants, the dissociation rate constants were determined for experiment I (8.46×10^{-3} min^{-1}) and II (6.69×10^{-3} min^{-1}). The values correspond to the rate at which the ligand dissociates from the receptor. No data has been published to date regarding the dissociation rate constant for the GnRH receptor. However, since the derivation of the constant is dependent on the change in surface-bound radioligand over time, the rate for the equine GnRH receptor may vary significantly from other GnRH receptors that undergo receptor downregulation.

Observations made from the electron microscopy experiment support the theory of rapid receptor recycling. Within ten minutes after initiating GnRH treatment, GnRH associated with receptor was detected within the gonadotropes (Figure 3-12) however, after 40 minutes free receptor was detected at the cell surface (Figure 3-13). Free receptor was detected on the cell surface at 60 minutes (Figure 3-14) and as late as 7 hours after initiating GnRH treatment (Figure 3-15). Although quantification of data was not feasible, a pattern of receptor internalization followed by quick receptor recycling was noted. Furthermore, the presence of receptor-ligand complexes at the cell surface after 7 hours may suggest a slow rate of internalization. These interpretations strengthen the data from the internalization experiments describing mechanisms, employed by the equine gonadotrope, to maintain gonadotropic responsiveness during continuous GnRH exposure.

GnRH receptor internalization is considered a potential component of receptor desensitization following ligand stimulation. This is in contrast to other G protein-coupled receptors that undergo desensitization of phosphatidylinositol hydrolysis. McArdle and co-workers (1995) reported that GnRH-stimulated desensitization did not occur via desensitization of phosphatidylinositol hydrolysis (McArdle CA et al., 1995). GnRH treatment of αT3-1 cells (murine gonadotrope cell line) resulted in a linear increase in total IP production and although there was a decline in IP-response following GnRH pretreatment, it was attributed to almost 50% reduction in cell surface GnRH receptor concentration following GnRH pretreatment. Cassina and co-workers (1997) reported similar declines in surface receptor number, in cultured rat pituitary cells treated with GnRH for 40 to 50 minutes (Cassina MP et al., 1997). In contrast to IP production, Anderson and co-workers did describe a GnRH-stimulated Ca^{2+} response which was desensitized in both αT3-1 and HEK-293 cell lines (Anderson L et al., 1995). Similarly, McArdle and co-workers (1995) reported that the GnRH-pretreatment caused a significant decline in Ca^{2+} response to subsequent GnRH challenge (McArdle CA et al., 1995). GnRH pre-treatment affected both the spike phase response (mobilization from intracellular IP_3-sensitive Ca^{2+} stores) and the plateau phase response (mobilization of extracellular Ca^{2+} via PKC-sensitive channels) of Ca^{2+} to GnRH treatment. Desensitization of the Ca^{2+} spike response was rapid (10-20 min) in onset whereas the recovery was slow (4-6 h). Moreover, in a study evaluating the factors that might be important in restoration of the desensitized gonadotrope, Gorospe and Conn (1988) demonstrated that recovery from desensitization might involve functional recovery of the voltage sensitive Ca^{2+} channels (Gorospe WC and Conn PM, 1988). The authors of these studies concluded that GnRH receptor desensitization might occur through several mechanisms, including a decline in surface receptor number and a reduction in ligand-stimulated intracellular Ca^{2+} mobilization.

The apparent lack of GnRH-induced IP desensitization in homologous cells lines expressing the mammalian GnRH receptor is unique compared to other G protein-coupled receptors. This may be due to the absence of a cytoplasmic terminal tail. The intracellular C terminus of most G protein-coupled receptors has been shown to be functionally important for G protein coupling (Cotecchia S et al., 1990), agonist-induced receptor internalization (Fukushima Y et al., 1997), and or Ser/Thr phosphorylation-mediated desensitization (Fukushima Y et al., 1997). Although mammalian GnRH receptor is lacking a C-terminal tail, receptor desensitization and internalization have been described suggesting a different mechanism from that used by other G protein-coupled receptors. In an attempt to better understand these differences, several studies have been performed using chimeric constructs of the mammalian GnRH receptor. Lin and co-workers (1998) added the catfish 51-amino acid intracellular C terminus to the rat GnRH receptor. This chimera bound to GnRH with the same affinity and significantly increased the surface receptor number (Lin X et al., 1998a). Additionally, inclusion of the catfish C-terminal domain resulted in an altered pattern of receptor regulation from biphasic down-regulation and recovery to monophasic downregulation of the rat GnRH receptor. Conn and co-workers first characterized the biphasic regulation of mammalian GnRH receptors by GnRH (Conn PM et al., 1984). In their study, mammalian GnRH receptors initially under went down-regulation (0.5-4 h post treatment) followed by upregulation of the receptor (9 h post treatment). This biphasic phenomenon has been described in wild type gonadotropes as well as cells transfected with mammalian GnRH receptor genes (Conn PM et al., 1984).

A similar study comparing the chicken and human GnRH receptor reported that receptor-mediated internalization of GnRH agonist was significantly faster for the chicken compared to the human receptor (Pawson AK et al., 1998). Furthermore, truncation of the chicken receptor such that the C-terminus was removed resulted in a rate of receptor internalization that was not different from that of the human GnRH receptor. Willars and co-workers (1999) demonstrated that the rat GnRH receptor does not undergo agonist-dependent phosphorylation (Willars GB et al., 1999). Furthermore, chimera construction by the addition of the C-terminus from the African catfish GnRH receptor resulted in a significant increase in agonist-dependent phosphorylation. Similar data, reported by Heding and co-workers (1998), demonstrated that chimera constructs involving the incorporation of the cytoplasmic tail from the rat TRH receptor into the rat GnRH receptor resulted in rapid desensitization of IP production and increased internalization rates (Heding A et al., 1998). These studies suggest that an alternative mechanism from that used by nonmammalian receptors may be present in mammalian

GnRH receptors. Moreover, species variation may exist among mammals such as the horse.

In summary, the rate of endocytosis for the equine GnRH receptor differed from those published in rats, mice, and sheep. During exposure to GnRH, the equine gonadotropes maintained a population of available receptors at the cell surface through rapid receptor recycling and/or insertion of newly synthesized receptors. This is in contrast to other mammals in which a significant decline in cell surface receptor has been described. These data represent the first description of a mechanism explaining the continuous LH response to constant GnRH secretion described in Equids.

CHAPTER 4
STEROIDAL REGULATION OF THE EQUINE GnRH RECEPTOR

Introduction

Equine GnRH receptor-internalization experiments (Chapter 3) indicated that mechanisms for maintaining adequate concentrations of GnRH receptors on the cell surface might exist in equids. These mechanisms may include a slow rate of receptor internalization accompanied by a fast rate of receptor recycling. An additional mechanism may include up regulation of the GnRH receptor gene. Up regulation of gene transcription and/or enhanced mRNA translation would increase the pool of GnRH receptors within the cytosol that may be inserted into the plasma membrane. Such up regulation has been reported in other species secondary to steroidal feedback. Ovine pituitary cells treated with estradiol responded with an increase in GnRH receptors, which was maintained for over 48 hours (Laws SC et al., 1990). Gregg and co-workers (1990) obtained similar data by treating primary cultures of ovine pituitary cells with estradiol, which resulted in dose-and time-dependent increase in number of GnRH receptors (Laws SC et al., 1990). Furthermore, actinomycin-D and cyclohexamide treatment abolished the estrogen-induced rise in GnRH receptor number suggesting that both transcription and translation are involved in estrogen's upregulation of the GnRH receptor. Similar data have been reported *in vivo.* Treatment of ovariectomized (OVX) ewes with estradiol resulted in a significant rise in GnRH receptor number and mRNA concentrations (Turzillo AM et al., 1998b). Moreover, expression of GnRH receptors increases prior to the onset of the preovulatory surge of LH in sheep. Several studies have demonstrated that this phenomena was due to declining progesterone levels following luteolysis and increasing estrogen concentrations from the preovulatory follicle (Turzillo AM et al., 1994; Kirkpatrick BL et al., 1998). Similar to sheep, in most mammals the preovulatory surge of GnRH is induced by increasing estrogen levels from the Graafian follicle (Kesner JS, 1988). Therefore, in sheep estrogen up regulates the GnRH receptor gene such that transcription and translation are enhanced resulting in increased cell-surface receptors facilitating the preovulatory surge of GnRH.

Comparable to other species, the peripheral concentrations of estrogen in mares increases during estrus prior to ovulation (Plotka ED, 1975). Additionally, increasing concentrations of estrogen are associated with increasing levels of GnRH and LH (Evans MJ and Irvine CHG, 1975) (Alexander SL and Irvine CHG, 1987). However, unlike other species, the duration of estrus in mares ranges between 5 to 7 days, noticeably longer than other domestic farm species (Hughs JP et al., 1972). Moreover, the pre-ovulatory surge of GnRH in the mare is better described as a *gradual rise* rather than a *surge*, peaking approximately 12 to 24 hours after ovulation. Endogenous GnRH secretion in mares during this time period has been described as irregularly episodic and continuous (Grubaugh WR and Sharp DC, 1987) (Alexander SL and Irvine CHG, 1987). GnRH secretion has also been described as continuous throughout the estrous cycle with intervening secretory events (Alexander SL and Irvine CHG, 1987). These studies suggest that GnRH secretion in mares during estrus is continuous thus a mechanism must exist within equine gonadotropes to maintain responsiveness to constant GnRH secretion. Estrogenic feedback on the equine gonadotrope remains a possibility however limited information exists regarding steroidal regulation of the equine GnRH receptor. Data from our laboratory indicated that long-term (10 days) administration of estradiol in ovariectomized pony mares resulted in elevated LH secretion but did not enhance the pituitary's response to GnRH challenge (Greaves H et al., 2000). Additionally, acute administration of estradiol to ovariectomized pony mares during the breeding season caused an increase in GnRH secretion 4-8 hr after estradiol administration (Porter MB et al., 1996). Data from equine pituitary cells in primary culture, reported an enhanced secretory response (LH) to GnRH in those cells pretreated with estradiol (Matteri RL et al., 1987). These studies suggest that estrogen's feedback on the hypothalamic-pituitary axis in horses may involve the pituitary gland and/or hypothalamus. However, the exact mechanisms have not been described.

Based on the lack of information regarding steroidal regulation of the equine GnRH receptor, the following experiments were proposed. In the first set of experiments (IA & AB), ovariectomized pony mares were treated with progesterone and estrogen and the effect on GnRH receptor expression was measured. In the second experiment, pituitary glands were collected from mares during estrus and diestrus to compare GnRH receptor number and mRNA concentrations during a progesterone-dominated versus an estrogen-dominated phase.

Materials and Methods

Experiment IA

Long-term ovariectomized pony mares were randomly assigned to two groups. Group 1 (n=3) received progesterone (75 mg twice daily) for 14 days followed by 7 days of vehicle injections. Likewise, Group 2 (n=3) was treated with progesterone (75 mg twice daily) for 14 days however progesterone treatment was followed by estrogen (5 mg once daily; beginning on day 15) treatment for 7 days in this group (Table 4-1). Blood samples were collected from mares daily for measurement of LH and progesterone concentrations via radioimmunoassay. On day 21, all mares were euthanized and pituitary glands collected for enzymatic dispersion. After enzymatic dispersion, pituitary cells were maintained in primary cell culture for 48 hours. Cells were cultured in non-adherent petri dishes containing DMEM media (Gibco DMEM pack™ (w/ Glucose, L-Glutamine, and Sodium Pyruvate) plus 25 mM Hepes, 0.25% BSA, 400 mg/L $CaCl_2$, 3.7 gm/L $NaHCO_3$, 10% heat-inactivated calf serum, and 1% antibiotic-antimycotic) under humidified conditions (37° C and 5% CO_2). Following 48 hr in primary cell culture, pituitary cells were washed in DMEM media (minus serum) and then challenged with 10^{-8} molar GnRH in DMEM (minus serum) for 3 hours. Pre- and post-GnRH challenge media was recovered to measure concentrations of LH.

Experiment IB

Long-term ovariectomized pony mares were randomly assigned to two groups. Group 1 (n=10) received progesterone (50 mg twice daily) for 14 days followed by 14 days of vehicle injections. Likewise, Group 2 (n=10) was treated with progesterone (50 mg twice daily) for 14 days however this group then received only vehicle injections for 4 days at which point they were treated with estrogen (5 mg once daily) for 7 days (Table 4-2). Both groups were challenged with GnRH on day 23 after beginning progesterone treatment. Prior to GnRH challenge, blood samples were collected every 30 minutes for 2 hours and after GnRH challenge every 15 minutes for 6 hours. Additionally, blood samples were collected daily from all mares for analysis of LH content via radioimmunoassay. On day 26 of the experiment, 3 mares from each group, selected at

Group 1: D1--------**(P4 Tx)**---------------D14   D15------**(vehicle)**-------------D21

Group 2: D1--------**(P4 Tx)**---------------D14   D15------**(E2 Tx)**---------------D21

Figure 4-1 Experimental protocol for experiment Ia. Pony mares were divided into two groups. Group 1 consisted of 3 OVX pony mares treated with progesterone (75 mg every 12 hours) for 14 days followed by injections of vehicle (sesame oil) for 7 days. Group 2 consisted of 3 OVX pony mares treated with progesterone (75 mg every 12 hours) for 14 days followed by estradiol (5 mg every 12 hours) treatment for 7 days. At the end of the experiment (day 21) all pony mares were euthanized and pituitary glands collected. Half of the pituitary glands were processed for cell culture and the remaining half frozen in liquid nitrogen for future binding studies. Throughout the 21 days of the experiment, blood samples were collected from all ponies to measure changes in circulating concentrations of LH.

Group 1: D1--**(P4 Tx)**--D14/D15--**(No Tx)**--D18/D19--**(vehicle)**--D23-**(vehicle)**-D26

Group 2: D1--**(P4 Tx)**--D14/D15--**(No Tx)**--D18/D19--**(E2 Tx)**--D23-**(E2 Tx)**---D26

Figure 4-2 Experimental protocol for experiment Ib. Pony mares were divided into two groups. Group 1 consisted of 10 OVX pony mares treated with progesterone (75 mg every 12 hours) for 14 days followed by no treatment for 4 days and then injections of vehicle (sesame oil) for 7 days. Group 2 consisted of 10 OVX pony mares treated with progesterone (75 mg every 12 hours) for 14 days followed by no treatment for 4 days, and then estradiol (5 mg every 12 hours) treatment for 7 days. On day 23 of the experiment all mares were challenged with a single dose of GnRH (100ug) and blood samples were collected at frequent time intervals. At the end of the experiment (day 26) 3 pony mares from each group were euthanized and pituitary glands collected. Half of the pituitary glands were processed for RNA extraction and the remaining half frozen in liquid nitrogen for future binding studies. Throughout the 26 days of the experiment, blood samples were collected from all ponies to measure changes in circulating concentrations of LH.

random were euthanized and the pituitary glands were removed. After removal, pituitary glands were bisected and quickly placed in liquid nitrogen. Half of the pituitary gland was for RNA extraction and the other half was for pituitary membrane preparations. Total RNA was extracted from pituitary glands and Northern Blot analysis was performed to quantify mRNA for the equine GnRH receptor. The protocol for preparing pituitary membrane samples is listed in Appendix B.

Experiment II

Experiment II was performed in collaboration with Dr. Luis Lossino and Dr. Lourdes Cordoba in Cordoba, Argentina, South America. Drs. Lossino and Cordoba evaluated mares daily via rectal palpation and ultrasound during the breeding season. Approximately 5 pituitary glands were collected from mares in estrus and 5 from mares in diestrus. Estrus was defined as showing sexual receptivity to a teaser stallion and the development of follicle, with a minimum of 30mm diameter. Records were kept regarding what stage (early, middle, or late) of estrus the mares were in at the time of euthanasia. Diestrus was defined as showing no sexual receptivity to the stallion and the presence of a corpus luteum as detected by trans-rectal ultrasound. Pituitary glands were harvested within 10 to 15 minutes after euthanasia and immediately immersed in liquid nitrogen. Tissues were maintained at –80° C until transport to our laboratory. Upon arrival, pituitary glands were bisected equally. Total RNA was extracted from one half and Northern Blot analysis performed to quantify concentrations of mRNA for the equine GnRH receptor. The remaining half was homogenized into pituitary membrane preparations to perform receptor-binding studies.

Pituitary Cell Dispersion Protocol

Pituitary glands were collected from horses within 15 minutes after euthanasia. The anterior pituitary was dissected free of the posterior pituitary and placed in sterile transport medium @ 4° C (25mM Hepes, 5mM KCl, 137mM NaCl, 10mM Glucose, 0.4% BSA and 1% antibiotic-antimycotic, pH = 7.4). After incubation at 4° C for 30-60 minutes, pituitary tissue was placed into a petri dish and cut into 5x5 mm (approximate) segments. The tissue fragments were then washed three times in cold transport medium and placed on ice for transport to the laboratory. In preparation for enzymatic dispersion, pituitary tissue fragments were further cut into 1 x 1 mm segments and then washed 3 times in cold transport medium. Tissue fragments were placed in a trypsinizing flask (150 ml) along with enzymatic dispersion solution (transport media (50ml), 0.25 % Collagenase Type II (Sigma C-6885), and 0.002 % DNAse (Sigma D-4263) @ 37° C). The flask was placed on a stir plate within an incubator set at 37° C for 5 minutes, after which the dispersion solution was removed (minus tissue fragments) and centrifuged to remove non-viable pituitary cells and red blood cells (RBC). Subsequently, the dispersion solution was added back to tissue fragments and allowed to incubate at 37° C for 10 minutes on a stir plate. After 10 minutes of stirring, the dispersion solution was removed (minus tissue fragments) and centrifuged to collect dispersed pituitary cells. The dispersion solution (minus dispersed pituitary cells) was added back to the tissue fragments for another 10 minutes. This was repeated 4-6 times or until all tissue fragments were completely dispersed. The recovered pituitary cells were washed 3 times in transport media at 37° C to remove RBC's and then passed through a 20 μM nylon filter (Fischer) to eliminate cellular debris. After dispersion was complete, total cell count and viability were determined using a hemocytometer and 0.04% trypan blue solution. The pituitary cell recovery averaged 20 to 50 million cells per pituitary gland with >90% viability.

RNA Extraction and Northern Blot Protocol

Total cellular RNA was prepared from equine anterior pituitary glands by the TRIzol™ method (Life Technologies, Inc). Equine anterior pituitary gland tissue (100 mg) was briefly homogenized in 1 ml of TRIzol™ reagent (phenol and guanidine isothiocyanate solution). Addition of chloroform followed by centrifugation, divided the solution into an aqueous phase, containing the RNA, and an organic phase. The aqueous phase was recovered and the RNA was precipitated from this solution with isopropanol. After precipitation, RNA was re-constituted in sterile water and total RNA quantified by measuring the absorbance at 260 nm.

For Northern blot analysis, approximately 30 μg of total RNA were separated on 1.0% agarose-2.2 M formaldehyde gels and gels transferred to a nitrocellulose membrane by downward capillary transfer using the TurboBlot system (Schleicher & Schuell, Keene, NewHamshire). Nitrocellulose membranes were cross-linked by brief (60 sec) exposure to ultraviolet light and the membranes were baked for 2 hours at 80^0C. The prehybridization and hybridization of the membranes was done using ULTRAhyb™ Buffer (Ambion) and the $^{32-}$P-labelled equine GnRH receptor cDNA probe was prepared by following the protocol as provided by the Nick translation kit (Amersham Pharmacia Biotech). Following overnight hybridization with the $^{32-}$P-labelled equine GnRH receptor cDNA, the membranes were subjected to several low and high stringency washes. The blots were then examined by autoradiography. Densitometric analysis of the developed bands on the autoradiograms was performed to quantify GnRH receptor mRNA. The blots were rehybridized with $^{32-}$P-labelled equine house-keeping gene cDNA probe GAPDH) and the densitometeric values representing GAPDH mRNA where used to adjust for differences in RNA loading.

Saturation Binding Experiments

Saturation binding experiments were performed using equine pituitary membrane preparations to estimate the dissociation constant (K_d) and receptor number (Bmax). The saturation binding experiments consisted of incubating equal concentrations of pituitary membranes with increasing amounts of radiolabelled GnRH. Non-specific binding (NSB) was determined at all concentrations of radiolabelled D-Alanine GnRH added by including samples with 100-fold excess of unlabelled D-Alanine GnRH. Total radioactivity (CPMs) was adjusted for NSB and then converted to picomolar (pM) concentrations based on the estimated specific activity (2754 CPM/picogram) of radiolabelled D-Alanine GnRH. Bound GnRH concentrations (pM) were plotted on the Y-axis and free GnRH concentrations (pM) were plotted on the x-axis. Data were fit to a saturation binding curve equation (one site binding) and the estimate of Bmax was based on the predicted concentration of bound radioligand which would equal the point of saturation. The K_d equaled the concentration of free radioligand at which bound is 50% of Bmax.

Supplies

Estradiol (β-Estradiol; Sigma Chemical E-8875) was solubilized in benzyl alcohol (Fischer Scientific A396-500) and diluted to desired concentration (1.7mg/ml) in sterile sesame oil (Sigma Chemical S-7131). Likewise, progesterone (4-pregnene-3, 20-dione; Sigma Chemical P-1030) was dissolved in benzyl alcohol (Fischer Scientific A396-500) and diluted to desired concentration (25mg/ml) in sterile sesame oil (Sigma Chemical S-7131). Natural GnRH (BACHEM Switzerland H-4005) was solubilized in sterile 0.9% saline and diluted to desired concentration in phosphate buffered saline (pH= 7.4). D-Alanine GnRH (Sigma Chemical L4513) was radioiodinated using a lactoperoxidase-Glucose Oxidase iodination (Appendix A).

Hormone Assays of Blood Samples

Approximately 8-10 ml of blood was collected into heparinized tubes and stored at 2° C until plasma was harvested via centrifugation. Equine LH was analyzed by radioimmunoassay using bovine antiserum to LH and purified equine LH provided by Dr. Janet Roser (University of California, Davis, California) (Matteri RL et al., 1987). The assay sensitivity, intra- and interassay coefficients of variation were 1.2 ng/ml, 4.5%, and 4.6%, respectively. Equine progesterone concentrations were measured in plasma using a commercial solid phase radioimmunoassay kit provided by Diagnostic Products Corp, Los Angeles, CA. The assay sensitivity, intra- and interassay coefficients of variation were 0.5 ng/ml, 4.3%, and 4.5%, respectively.

Statistical Analysis

Data were analyzed using Statistical Analysis System (SAS) by least squares analysis of variance (ANOVA). Main effects of treatment and treatment-by-day interactions were tested for significance and time response curves representing daily samples for each treatment were analyzed for homogeneity of regression. In addition to analysis of daily LH concentration, LH levels measured during the frequent sampling period on day 23 were analyzed by ANOVA. Main effects of treatment and treatment-by-sample interactions were tested for significance. Furthermore, regression analyses were performed on LH time trends. Densitometric data from Northern Blots were analyzed using SAS by ANOVA and main effects of treatment were tested for significance.

Results

Experiment IA

LH concentrations declined in both groups following the onset of progesterone treatment and remained low until the withdrawal of progesterone (Figure 4-3). In group 1 mares, LH levels returned to pre-treatment levels within 4 to 5 days after stopping progesterone treatment. In contrast, LH concentrations in group 2 mares remained low throughout estradiol treatment (Figure 4-3). Regression analysis indicated that the time trend curves representing daily LH were not parallel ($p<0.01$) due to the variation in LH levels post-progesterone treatment. In addition to suppressed LH secretion in group 2 mares, pituitary cells collected from these mares had a significantly reduced response to GnRH in culture. Pituitary cells from mares treated with progesterone-only responded with an almost 3-fold increase in LH release whereas pituitary cells from group 2 mares responded minimally to GnRH challenge in culture (Figure 4-4). The persistent suppression of LH secretion in mares treated with estradiol immediately after 2 weeks of progesterone treatment was not expected. We speculated that there might have been residual amounts of progesterone circulating at the time of estradiol treatment therefore; progesterone levels were measured in blood samples collected during the seven days after terminating progesterone treatment. Interestingly, progesterone concentrations did not decline for 5 to 6 days even though treatment had been stopped (Figure 4-5). Therefore, the experiment was re-designed and repeated as experiment IB.

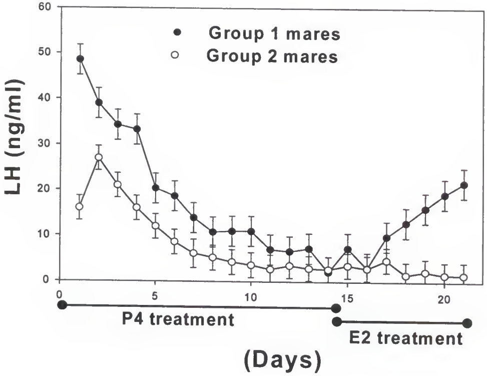


Figure 4.3 Daily time trends representing least square means for daily LH concentrations in experiment IA. Mares in group 1 were treated with progesterone (P4) for 2 weeks followed by vehicle for 7 days. In contrast, group 2 mares were treated with P4 for 2 weeks followed by estrogen treatment of 1 week. LH concentrations declined in both groups during the progesterone treatment. After P4 treatment was terminated in group 1 mares, circulating levels of LH began to rise and returned to pre-treatment levels. However, mares treated with estradiol following P4 treatment continued to have low LH levels. Regression analysis indicated that the curves were not parallel and this was due in part to the persistent suppression of LH in group 2 mares.

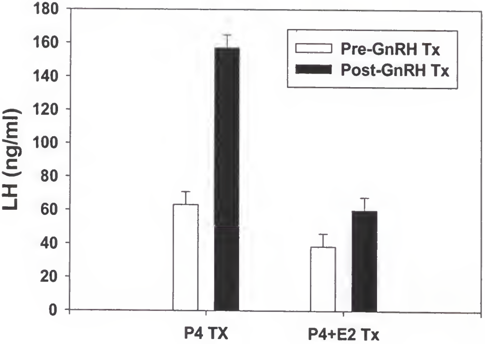


Figure 4.4 Least square mean values representing the LH secretory responses to GnRH challenge in dispersed equine pituitary cells in experiment IA. Pituitary glands were collected from ponies at the end of the experiment (day 21) and dispersed via an enzymatic solution (Collagenase) for cell culture purpose. After 48 hours in static culture, pituitary cells were exposed to culture media containing 10^{-8} M GnRH for 3 hours. After 3 hours, culture media was collected and stored at -20° C until LH concentrations were measure via radioimmunoassay.

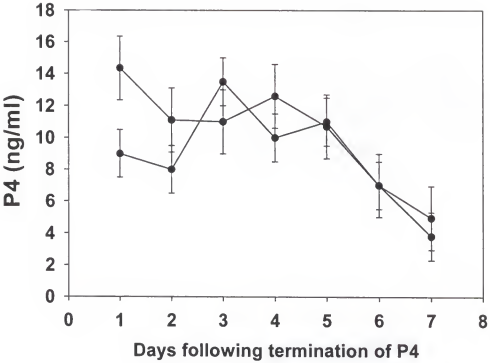


Figure 4-5 Least square mean values representing circulating progesterone concentrations in daily samples collected from both groups after termination of progesterone treatment in experiment IA. Although P4 treatment had ended, high levels (> 1ng/ml) of progesterone were detectable in circulation. These concentrations persisted for nearly 1 week after termination of progesterone treatment. Based on these data, a progesterone recovery period was instituted in experiment IB.

Experiment IB

As in experiment IA, LH concentrations declined and remained low throughout progesterone treatment in both groups of mares (Figure 4-6). Once progesterone treatment was stopped, LH levels began to return to original levels in control mares. Likewise, LH levels in group 2 mares paralleled group 1 mares for the 4 days following the end of progesterone treatment. However, LH levels in mares treated with estradiol continued to rise throughout estradiol treatment and were maintained at significantly higher concentrations than group 1 mares (Figure 4-6). As in experiment IA, regression analysis of LH time trends indicated that the curves were not different and this could be attributed to the increased LH secretory response in group 2 mares. Analysis of LH time trends before and after GnRH challenge on day 23 indicated that both groups responded to GnRH with a rise in LH concentrations however the amplitude of response was not different between groups (Figure 4-7).

Analysis of variance performed on receptor binding studies indicated that there was a significant ($p<0.05$) increase in radioligand binding in pituitary membrane preparations from group 2 mares compared to group 1 mares (Figure 4-8). This increase in binding corresponded to a 10-fold increase in receptor affinity in pituitary glands treated with progesterone followed by estradiol compared to those treated to progesterone alone (Figure 4-12). Interestingly, there was a 10-fold decline in cell surface receptor number in group 2 mares versus group 1 mares. Additionally, Northern Blot analysis revealed a significant ($p<0.05$) decline in GnRH receptor mRNA in group 2 mares compared to group 1 mares (Figure 4-9).

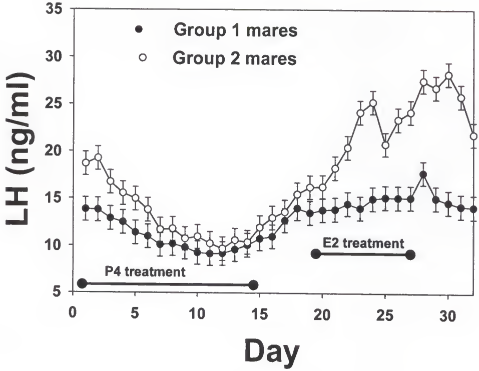


Figure 4.6 Daily time trends representing least square means for daily LH concentrations in experiment IB. Mares in group 1 were treated with progesterone (P4) for 2 weeks followed no treatment for 4 days and then vehicle (sesame oil) for 7 days. In contrast, group 2 mares were treated with P4 for 2 weeks followed by no treatment for 4 days and then estrogen (5 mg/12 hrs) treatment of 1 week. LH concentrations declined in both groups during the progesterone treatment. After P4 treatment was terminated in group 1 and 2 mares, circulating levels of LH began to rise in a similar fashion. However, mares treated with estradiol following P4 treatment responded with a continued rise in LH levels, above those measured in control mares treated with vehicle alone. Regression analysis indicated that the curves were not parallel and this was due in part to the continued rise LH levels in group 2 mares treated with estradiol.

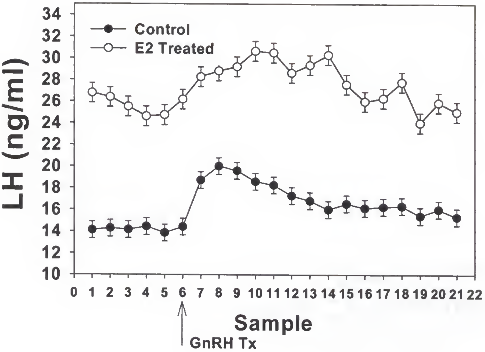


Figure 4-7. Least square mean values representing LH concentrations before and after GnRH challenge on day 23 of experiment IB. Blood samples were collected every 30 minutes for 2 hours before GnRH challenge and then every 15 minutes for six hours after GnRH treatment. All mares were treated with 100 ug of GnRH. Immediately after GnRH administration, LH levels increased in both groups of mares and analysis of variance indicated that there was no difference between groups in LH response to the GnRH. Furthermore, homogeneity of regression indicated that these curves were not different. Although the baseline levels were significantly higher in mares being treated with estrogen, the amplitude of the LH response to GnRH did no differ between groups.

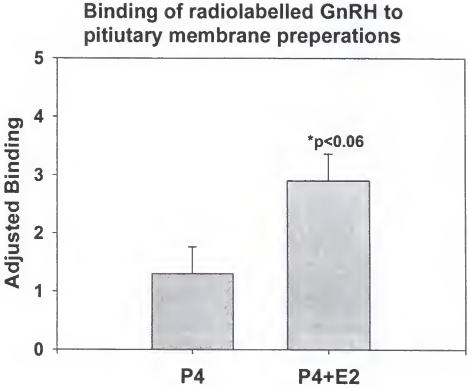


Figure 4-8 Least square means representing the percent binding (%) measured in membrane preparations from equine pituitary glands. Pituitary glands were collected from group 1 mares (P4) and group 2 mares (P4+E2). Approximately 1 mg of protein (membrane preparation) was combined with radiolabelled D-Alanine GnRH along with parallel samples containing excess "Cold" D-Alanine GnRH for measurement of non-specific binding. In addition to adjusting for non-specific binding, binding was adjusted for concentration of protein in each sample. Analysis of variance indicated that there tended ($p<0.06$) to be more binding in membrane preparations from pituitary glands in mares treated with P4 for 14 days followed by estradiol for 7 days.

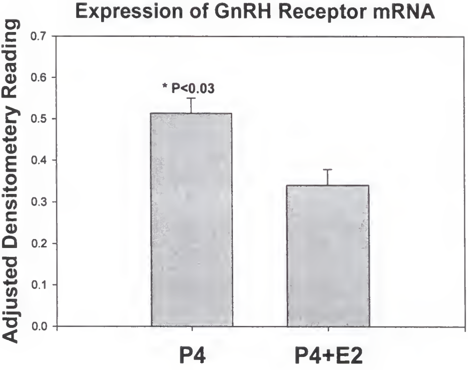


Figure 4-9 Least square means representing the concentration of mRNA as expressed in arbitrary densitometeric units. Pituitary glands were collected from group 1 mares (P4) and group 2 mares (P4+E2) followed by RNA extraction. Approximately 30 ug of total RNA were separated on 1.0% agarose-2.2 M formaldehyde gels and gels transferred to a nitrocellulose membrane. The nitrocellulose membranes were then hybridized with a $^{32-}$P-labelled equine GnRH receptor cDNA probe. The blots were then examined by autoradiography. Densitometric analysis of the developed bands on the autoradiograms was performed to quantify GnRH receptor mRNA. The blots were rehybridized with $^{32-}$P-labelled equine house-keeping gene cDNA probe (GAPDH) and the densitometeric values representing GAPDH mRNA where used to adjust for differences in RNA loading. Analysis of variance indicated that there was ($p<0.03$) greater expression of the equine GnRH receptor gene in RNA extracted from pituitary glands in mares treated with P4 for 14 days followed by vehicle treatment compared to those mares treated with estradiol for 7 days after P4 treatment.

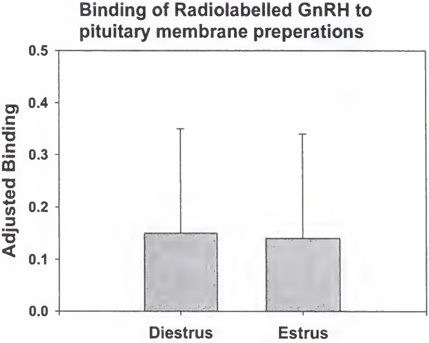


Figure 4-10 Least square means representing the percent binding (%) measured in membrane preparations from equine pituitary glands. Pituitary glands were collected from mares in estrus and diestrus. Approximately 100 µg of protein (membrane preparation) was combined with radiolabelled D-Alanine GnRH along with parallel samples containing excess "Cold" D-Alanine GnRH for measurement of non-specific binding. In addition to adjusting for non-specific binding, binding was adjusted for concentration of protein in each sample. Analysis of variance indicated that there were no differences in binding between membrane preparations from mares in estrus versus diestrus.

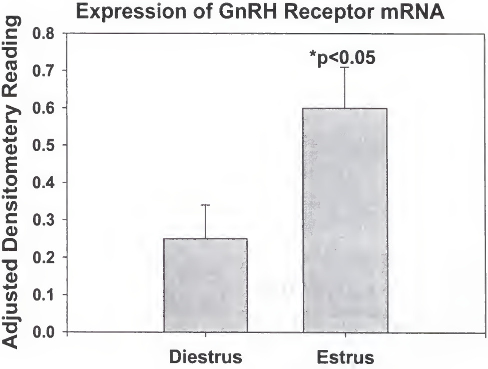


Figure 4-11 Least square means representing the concentration of mRNA as expressed in arbitrary densitometeric units. Pituitary glands were collected from mares in estrus and diestrus followed by RNA extraction. Approximately 30 ug of total RNA were separated on 1.0% agarose-2.2 M formaldehyde gels and gels transferred to a nitrocellulose membrane. The nitrocellulose membranes were then hybridized with a $^{32-}$P-labelled equine GnRH receptor cDNA probe and the blots were then examined by autoradiography. Densitometric analysis of the developed bands on the autoradiograms was performed to quantify GnRH receptor mRNA. The blots were rehybridized with $^{32-}$P-labelled equine house-keeping gene cDNA probe (GAPDH) and the densitometeric values representing GAPDH mRNA where used to adjust for differences in RNA loading. Analysis of variance indicated that there was ($p<0.05$) a significant increase in concentration of mRNA for the equine GnRH receptor in pituitary glands from mares in estrus versus diestrus.

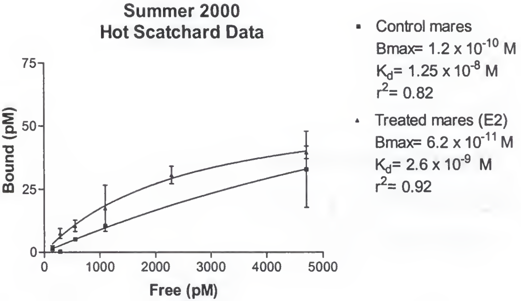


Figure 4-12 Non-linear regression analysis for the equilibrium binding of equine GnRH receptor in dispersed equine pituitary cells from control mares and mare treated with estradiol. Equal quantities (100 ug) of pituitary membrane preparations were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for non-linear regression. The data were entered into a one site binding equation also known as a saturation binding curve and the K_d and Bmax were calculated by determining the point of saturation (Bmax) and the molar concentration of free radioligand at 50% of Bmax, which is the K_d. These data indicate a 10-fold increase in affinity in pituitary tissues from mares treated with estradiol for 7 days after P4 treatment. However, there appeared to be 10-fold fewer binding sites in pituitary tissues from mares treated with estradiol compared to mares treated with vehicle.

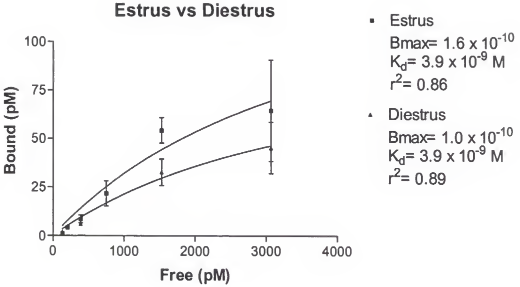


Figure 4-13 Non-linear regression analysis for the equilibrium binding of equine GnRH receptor in dispersed equine pituitary cells from control mares and mare treated with estradiol. Equal quantities (100 µg) of pituitary membrane preparations were incubated at 4° C for 4 hours with increasing concentrations of ^{125} D-Alanine GnRH. Surface bound radioligand was separated from free ligand by centrifugation and non-specific binding was subtracted from total binding to reveal specific binding. The binding in counts per minute was converted to molar concentrations of radioligand for non-linear regression. The data were entered into a one site binding equation also known as a saturation binding curve and the K_d and Bmax were calculated by determining the point of saturation (Bmax) and the molar concentration of free radioligand at 50% of Bmax, which is the K_d. These data indicate no change in affinity for GnRH or GnRH receptor number between mares treated with estradiol after P4 treatment and mares treated with vehicle after P4 treatment.

Experiment II

Analysis of variance performed on data from receptor binding studies indicated that there was no difference in receptor affinity or receptor number in pituitary membrane preparations from mares in estrus versus diestrus (Figure.4-10). This was due to a lack in difference in receptor affinity and receptor number between mares in estrus versus diestrus (Figure 4-13). However, Northern Blot analysis revealed a significant ($p<0.05$) increase in GnRH receptor mRNA concentration in the pituitary glands from mares in estrus (Figure 4-11). Retrospective examination of the estrus stage of mares at the time of pituitary collection revealed that 3 out 5 were in early estrus whereas the 2 remaining were in late estrus Insufficient animal numbers precluded statistical comparisons between mares in early estrus versus late estrus however those mares in late estrus appeared to have the highest concentrations of GnRH receptor mRNA.

Discussion

The GnRH receptor gene is influenced by a variety of hormones including, steroids, gonadal peptides, second messenger activators and GnRH itself. Estradiol appears to have a species-specific regulation of GnRH receptor expression. In αT3-1 cells, estradiol caused a reduction in GnRH receptor number however in rat pituitary cells both an increase and decrease in GnRH receptor number were documented (Emons G et al., 1988; McArdle CA et al., 1992). *In vivo* experiments in rats which were ovariectomized and then treated with estradiol 21 days later, reported a significant decline in GnRH receptor mRNA by 7 days (Kaiser UB et al., 1993). In contrast, ovine pituitary cells treated with estradiol responded with an increase in GnRH receptors, which was maintained for over 48 hours (Laws SC et al., 1990). Gregg and co-workers (1990) obtained similar data by treating primary cultures of ovine pituitary cells with estradiol, which resulted in dose-and time-dependent increase in number of GnRH receptors (Laws SC et al., 1990). Furthermore, actinomycin D and cyclohexamide treatment abolished the estrogen-induced rise in GnRH receptor number suggesting that both transcription and translation were involved in estrogen upregulation of the GnRH receptor. Hence, the estradiol regulatory effect on ovine GnRH receptor expression may be genomic. *In vivo* data from orchidectomized sheep reported a significant, dose-dependent rise in tissue levels of GnRH receptor mRNA following infusion with estradiol, which was dose-dependent (Gregg DW et al., 1990) (Sakurai H et al., 1997). Furthermore, withdrawal of estrogen treatment resulted in a rapid decline in concentrations of GnRH receptor mRNA (Adams BM et al., 1996). The withdrawal of estrogen is also associated with a reduction in the concentration of GnRH receptors. This decline in receptor number suggests that estrogen withdrawal may destabilize GnRH receptor mRNA and/or may reduce the rate of transcription (Sakurai H et al., 1997).

The effect of the other gonadal steroid, progesterone, appears to be inhibitory on the expression of GnRH receptors. Data obtained from *in vivo* rat studies revealed a significant increase in GnRH receptor mRNA due to estrogen treatment of ovariectomized rats, however progesterone treatment decreased steady state levels of GnRH receptor mRNA (Adams BM et al., 1996). Additionally, Sealfon and co-workers (1990) reported an inhibitory effect of progesterone on GnRH receptor concentration when ovine pituitary cell in primary culture were treated with progesterone (Bauer-Dentoin AC et al., 1995). More recent data from studies evaluating the effect of progesterone treatment in orchidectomized sheep, indicated that pituitary tissue concentrations of GnRH receptor number and mRNA were significantly reduced in wethers implanted with progesterone (Sakurai H et al., 1997). The inhibitory effect of progesterone on pituitary GnRH receptor mRNA was corroborated by Wu and co-workers (1994). Treatment of ovine pituitary cell cultures with progesterone resulted in a 50% reduction in levels of GnRH receptor mRNA (Wu JC et al., 1994). Greg and co-workers reported that treatment of ovine pituitary cells with progesterone plus estradiol resulted in an additive increase in GnRH receptor number. However, progesterone alone did not cause an increase in receptor number suggesting that progesterone may potentiate estradiol's ability to increase receptor number.

The decline in LH secretion during progesterone treatment, which was described in both experiment IA and IB (Figure 4-3), was anticipated based on the known inhibitory effect of progesterone on LH secretion (Porter MB et al., 1997b). Furthermore, once progesterone treatment was halted and the negative feedback on the pituitary gland was removed, LH concentrations gradually returned to pretreatment levels. However the continued suppression of LH secretion in group 1 mares (experiment IA) was not anticipated (Figure 4-3). Previous data from this laboratory have demonstrated a positive feedback of estrogen on LH secretion, as is the case in most mammals (Greaves H et al., 2000). However, if estrogen is combined with progesterone, a profound negative feedback on LH secretion occurs in pony mares (Sharp DC et al., 1991). These data prompted us to measure progesterone concentrations in blood samples collected daily from both groups after progesterone treatment had been stopped. Surprisingly, elevated levels of progesterone were present in the periphery for up to 6 days after terminating progesterone treatment (Figure 4-5). Furthermore, dispersed pituitary cells from mares treated with estradiol immediately after 2 weeks of progesterone treatment, had a significantly reduced response to GnRH in cell culture (Figure 4-4). These data suggest that the presence of progesterone during estrogen treatment reduced the pituitary's ability to respond to endogenous or exogenous GnRH. However, experiment IA did not provide any information regarding the direct effects of progesterone and estrogen on the equine GnRH receptor.

Therefore, experiment IB was designed so as to allow time for metabolic clearance of progesterone and to address the effects of progesterone and estrogen on the equine GnRH receptor. Prior to the onset of estradiol treatment in experiment IB, progesterone concentrations were measured in blood samples collected during the 4 days after terminating progesterone treatment. These analyses indicated that by day 4, progesterone levels were below 1 ng/ml in both groups (data not shown), allowing for the initiation of estrogen treatment. In contrast to experiment IA, LH levels in group 1 and 2 rose in parallel until estrogen treatment began in group 2 mares (Figure 4-6). At this point, the time trends deviated due to the apparent accelerated increase in LH secretion in mares treated with estradiol. LH concentrations in control mares returned to pre-treatment levels and remained as such for the duration of the experiment. However, LH levels in group 2 mares continued to rise and were maintained at higher concentrations compared to those in group 1 mares (Figure 4-6). On the day of GnRH challenge, both groups of mares responded with an increased secretory response to GnRH (Figure 4-7). The absolute levels were greater in group 2 mares however; the amplitude of LH response was not different between groups. This may have been due to decreased LH stores within gonadotropes of group 2 mares due to the persistently elevated levels of LH secretion.

The 10-fold increase in receptor affinity and the 1-fold decrease in receptor number in pituitary membrane preparations from group 2 mares suggest several possibilities. The decline in available receptor number may be due to elevated levels of endogenous GnRH secondary to estrogen treatment. Previous work in our laboratory demonstrated an increase (4-8 h) in GnRH secretion following acute estradiol treatment (Porter MB et al., 1996). Thus there may be available GnRH receptors because of enhanced GnRH exposure. The increase in receptor affinity might involve a mechanism for adjusting to the reduced receptor number. Interestingly, the binding increased in pituitaries from estrogen-treated mares (group 2) indicating that the increase in affinity surpassed the decline in receptor number (Figure 4-8). However, although the binding was greater in pituitary membranes from mares treated with estradiol, the levels of GnRH receptor mRNA were lower in group 2 mares (Figure 4-9). A negative effect of estradiol on GnRH receptor number and mRNA has been reported in αTR-1 cells and dispersed rat pituitary cells (Emons G et al., 1988; McArdle CA et al., 1992).

The data from *in vitro* experiments and *in vivo* experiments in ovariectomized or castrated animals are supported by hormonal changes taking place throughout the estrous cycle. During the periovulatory period, increasing estradiol secretion from the preovulatory follicle results in increased concentration of pituitary GnRH receptor (Adams BM et al., 1996). In ewes, tissue concentrations of both GnRH receptor and GnRH receptor mRNA increase with increasing endogenous estradiol (Sakurai H et al., 1997). The concentration of GnRH receptor mRNA in sheep is significantly higher during the estrus phase as compared to diestrus. In one study, concentrations of GnRH receptor mRNA increased significantly within 12 hours after the induction of luteolysis

(Turzillo AM et al., 1994). This increase in GnRH receptor mRNA was associated with declining progesterone levels and occurred before measurable changes in circulating estradiol concentrations. Turzillo and Nett (1999) suggested that the declining levels of progesterone, due to luteolysis, initiate the molecular events responsible for increased concentrations of GnRH receptor mRNA. Subsequently, as the pre-ovulatory follicle matures, rising levels of estradiol may function to maintain elevated GnRH receptor mRNA and receptor number (Turzillo AM and Nett TM, 1999). The increasing level of GnRH receptor mRNA is maximal just before ovulation and drops significantly at the time of the GnRH surge (Cowley MA et al., 1998). Similar data have been reported during the rat estrous cycle (Clayton RN et al., 1980). These physiologic data support the theory that estradiol up regulates the expression of GnRH receptors during the periovulatory period whereas progesterone may inhibit the expression of the GnRH receptor during the luteal phase. The pre-ovulatory upregulation of pituitary GnRH receptor heightens the sensitivity of the anterior pituitary gland to GnRH in preparation for the pre-ovulatory GnRH surge and ovulation.

Similar observations were made when mRNA concentrations from mares in estrus were compared to those in diestrus. Although there was a significant increase in mRNA concentration in pituitaries during estrus, there was no difference in receptor binding to radioligand between estrus and diestrus (Figure 4-10 and 4-11). The lack of difference in binding might have represented a lag between increased transcription and translation. Furthermore, investigations into the differences between early estrus and late estrus need to be performed. Especially considering that GnRH does not reach maximum levels until

12 to 24 hours after ovulation, it remains possible that GnRH mRNA levels and receptor number vary significantly from early estrus to the time of ovulation.

In summary, these data suggest that estrogen and progesterone play an important role in regulating the gonadotropic response to GnRH in horses. In general, progesterone suppresses LH secretion and appears to maintain basal GnRH receptor mRNA levels during diestrous. However, specific conclusions cannot be made with respect to a direct effect of progesterone on receptor number and affinity. In contrast, estrogen appears to up regulate the GnRH receptor during estrus, however, this may be in concert with GnRH itself. The administration of estrogen, after 2 weeks of progesterone treatment, significantly increased LH secretion and GnRH receptor affinity; however it prompted a suppression of GnRH receptor mRNA. These data conflict with the data from estrus mares indicating that the progesterone-primed, estrogen-treated mares may not be ideal models for studying the periovulatory regulation of the GnRH receptor.

GENERAL DISCUSSION

Early work from our laboratory clearly described distinct qualities of the equine hypothalamic-pituitary axis compared to that of other domestic species. These differences involved the gonadotropic response to GnRH and the ability of gonadotropes to maintain sensitivity to continuous GnRH treatment. Although the *in vivo* data indicated that the unique physiologic phenomena existed in horses, there was no information regarding *how* it occurred. Therefore, we proposed to describe the equine GnRH receptor and evaluate its physiologic behavior. The results from this work have helped explain the mechanisms behind the insensitivity of equine gonadotropes to GnRH receptor downregulation.

In Chapter 2, the equine GnRH receptor was cloned and sequenced. Comparative analysis between the deduced amino acid sequence in horses versus that of other species indicated a high (>85%) degree of homology. More importantly, amino acid residues, which have been identified as essential for proper function, have been conserved in the equine GnRH receptor. However, several amino acids in the equine GnRH receptor are unique in location along the protein structure. Although there are only 5 distinct amino acids, several of them are located in key regions of the receptor such as the ligand binding domain (N-terminus) and the G-protein binding domain (intracellular loops). Furthermore, there is extensive research indicating that in species examined a single point mutation may impart structural changes affecting the physiologic behavior of the receptors. Considering the *in vivo* data in horses, variations in the ligand-binding domain may affect the method with which the equine GnRH receptor binds GnRH. It is possible that the equine GnRH receptor is able to dissociate from GnRH once internalized at a quicker rate compared to other species due to differences in amino acid structure. This would account for an increase in available receptor for recycling to the cell surface. Alternatively, amino acid residue variation in the G-protein binding domains may alter the efficiency and/or duration of G-protein signaling. It is known that dissociation of the G-protein from the receptor initiates the process of desensitization and receptor-ligand internalization. Therefore, continued G-protein association with receptor may minimize the development of those signal-attenuating conditions. Clearly, continued research is indicated consisting of point mutation studies evaluating the significance of the unique amino acid residues in the equine GnRH receptor.

Most of the data regarding the trafficking of the GnRH receptor comes from transfected cell lines or immortalized cell lines, which are expressing the GnRH receptor. Unfortunately, an immortalized cell line of equine origin, which is expressing the GnRH receptor, does not exist. However, transfection of the equine GnRH receptor gene into a gonadotropic cell line from other species would be important to determine if the unique trafficking properties are receptor-dependent, gonadotrope-dependent, or species dependent. Additionally, transfection of the equine GnRH gene, which has been mutated, would provide an opportunity to determine the importance of the unique amino acid residues.

In Chapter 3, the physiologic behavior of the equine GnRH receptor was characterized. The values for the receptor affinity and cell surface number agreed with that of other species indicating that, in general, the equine GnRH receptor binds GnRH with the same affinity as that of other mammalian GnRH receptors. Furthermore, similar receptor population densities exist in unstimulated equine gonadotropes versus gonadotropes in other species. However, when equine gonadotropes were stimulated with GnRH, a distinct physiologic behavior was noted. GnRH challenge *in vitro* resulted in a steady increase in cell-surface binding in equine gonadotropes. This is in stark contrast to other species in which cell-surface binding declines due to receptor-ligand internalization. Derivation of GnRH receptor rate constants indicated that the equine GnRH receptor internalized at a significantly slower rate compared to that of other species. Observations from the electron microscopy images indicated that although the receptor-ligand complexes were observed in the cell interior, and therefore likely underwent internalization, receptor was observed at the cell surface within a short time plus some receptor-ligand complexes were maintained at the cell surface for up to 7 hours. Overall, these data suggest that equine gonadotropes maintain sensitivity to GnRH by keeping sufficient numbers of receptors at the cell surface. This is not to say that equine gonadotropes are not susceptible to downregulation or desensitization. On the contrary, at super-physiologic concentrations of GnRH or GnRH agonists, equine gonadotropes are likely to be incapable of maintaining adequate number of cell surface receptors. However, this has not been reported to occur in naturally occurring physiologic conditions. This is fortunate considering the prolonged period of estrus in mares during which GnRH concentrations are steadily rising. Although it appears that the equine GnRH receptor recycles to the cell surface quickly, the mechanism(s) regulating the process of recycling have not been described. GnRH stimulation of equine gonadotropes may result in the upregulation of cytosolic factors that facilitate the receptor recycling process however this is not known. Therefore, future research is indicated regarding the factors that are involved in receptor recycling.

In addition to potential cytosolic factors regulating GnRH receptor behavior, gonadal steroids such as estrogen and progesterone have been implicated in the regulation of GnRH receptor gene expression in other species. In the final set of experiments, the effect of estrogen and progesterone-withdrawal were evaluated in pony mares. Interestingly, although there was an increase in receptor binding in those mares (Group 2) treated with estradiol after a progesterone treatment, there was a decline in GnRH receptor gene expression in these same mares. Scatchard plot analysis indicated that the increased receptor binding was due to an increase in affinity in pituitary glands from mares treated with estradiol. However, there was a decline in receptor number that agreed with the decline in GnRH receptor gene expression. The decline in GnRH receptor gene expression was not expected considering the wealth of data in other species indicating that estrogen upregulates the GnRH receptor gene. A potential explanation may involve the 14 days of progesterone treatment prior to estrogen treatment. In the initial experiment, estrogen treatment started immediately after terminating progesterone treatment and the estrogen-induced increase in LH secretion was blocked. In the second experiment, 4 days were allowed for clearance of progesterone and the estrogen-induced rise in LH was noted. It is possible that although the gonadotropins were capable of responding to estrogen, residual progesterone levels interacted with estrogen to suppress GnRH receptor gene expression. This is supported by the data from mares in estrus in which expression of the equine GnRH receptor gene was upregulated. The significant increase in GnRH receptor mRNA concentrations during estrus appeared to be due to the two mares which were in late estrus. During this time period (last estrus) circulating progesterone are undetectable thus eliminating a negative feedback on the hypothalamic-pituitary axis by progesterone. Furthermore, rising concentrations of GnRH might potential contribute to positive feedback on expression of the GnRH receptor gene. This phenomenon is well documented in several species in which rising GnRH concentrations up regulate the GnRH receptor gene. Thus, endocrine conditions of progesterone-primed OVX mares treated with estradiol may not be identical to that of mares in late estrus. In particular, GnRH concentrations may be higher in late estrus mares compared to the progesterone-primed OVX mares treated with estradiol and peripheral progesterone levels may be lower in last estrus mares. It may be necessary to prolong the interval between the cessation of progesterone treatment and the onset of estrogen treatment. Alternatively, rising estrogen concentrations may not be as critical to GnRH receptor expression compared to rising GnRH concentrations. Regardless, the prolonged period of estrus in horses suggests a preparatory period that probably involves multiple factors in anticipation of ovulation. Clearly, the role of estrogen during the peri-ovulatory period of mares has not been completely elucidated, thus additional research in this area is indicated.

In conclusion, valuable insight has been gained regarding the hypothalamic-pituitary axis of mares. Data from these studies represent the first description of the physiologic behavior of the equine GnRH receptor, however continued research is necessary to complete the puzzle.

APPENDICES A
EQUINE PITUITARY MEMBRANE PREPARATION

1) Collect anterior pituitary and freeze immediately in liquid nitrogen

2) Thaw tissue on ice and weigh tissue (Approximately 1gm/pituitary)

3) Chop tissue finely with scalpel blade in petri dish while on ice

4) Transfer to a 20 ml glass tube and ad 10X volume homogenization Buffer (HB) (ex. 1 gm tissue plus 10 mls HB)

4) Homogenize thoroughly with a polytron homogenizer with sample on ice

5) Transfer to a 50ml centrifuge tube and rinse polytron with same volume of HB

6) Spin at 30,000 X G (16,000 rpm in a sorvall) for 30 minutes at 4 C

7) Scrape the top fluffy pink layer off of the pellet with a weighing spatula leaving the bottom white layer

8) Place in a small dounce homogenizer with 1 ml receptor assay buffer (RAB)

9) Homogenize on ice until no more clumps (8-10 strokes)

10) Transfer to a 12X75 tube plus rinse dounce with 1 ml RAB and add to tube

11) Spin at 30,000 X G (16,000 rpm in a sorvall) for 30 minutes at 4 C

12) Scrape the top fluffy pink layer off as in step 8

13) Place in small dounce homogenizer with 0.5ml RAB and homogenize (8-10X)

14) Transfer to new 12X75 tube and rinse dounce with 0.5 ml RAB (final Volume=1ml)

15) Determine protein concentration using Bio-rad kit and store at –70 C in 300ul aliquots.

APPENDICES B

GnRH Iodination

1) Add GnRH (D-Alanine GnRH Sigma L-4513) to reaction vial
   a. 10 ul (1 ug/ul in 0.2 M PO_4, pH 7.1)
2) Add 40 ul of 0.2 M PO4, pH=7.1 to reaction vial
3) Add Lactoperoxidase (Sigma L-8503) to reaction vial
   a. 1.5 ul (1ug/ul in ddH_2O)
4) Add Glucose solution to reaction vial
   a. 25 ul of a 0.1% Glucose solution in 0.1 M PO_4.
5) Add 2 miC of I_{125} to reaction vial and stopper
6) Add Glucose oxidase (Sigma G-6125) to reaction vial through rubber stopper
   a. 4 ul (1200 units/ml)
7) React for 5 minutes with gentle agitation
8) Stop reaction by adding 200 ul of 0.1 M Tris buffer
9) Elute column (QAE Sephadex Sigma -25-120) with Tris buffer (10mM Tris, 0.1% BSA, pH 9.2)
10) Count 36, 1 ml fractions

APPENDICES C

ANOVA TABLES CHAPTER 4

Experiment IA: Daily LH

Source	DF	Type IV SS	Mean Square	F value	PR>f
G	1	18.61	19.00	0.87	0.42
M(G)	3	64.00	22.00	2.97	0.05
S	6	233.4	39.00	5.41	0.002
G*S	6	51.0	8.43	1.17	0.36
ERROR	18	129	7.20		

Experiment IB: Daily LH

Source	DF	Type IV SS	Mean Square	F value	PR>f
G	1	1372	1372.0	1.55	0.230
M(G)	14	123998	884.0	81.0	0.001
S	26	4689	180.0	17.0	0.001
G*S	26	906	35.0	3.2	0.001
ERROR	358	3881	10.84		

Experiment IB: GnRH challenge

Source	DF	Type IV SS	Mean Square	F value	PR>f
G	1	7111	7111.0	4.00	0.070
M(G)	10	17792	1779.0	423.0	0.001
S	20	754	37.7	8.97	0.001
G*S	20	117	5.8	1.39	0.132
ERROR	196	824	4.2		

LITERATURE CITED

Adams BM, Sakurai H and Adams TE (1996). "Concentrations of Gonadotropin-Releasing Hormone (Gnrh) Receptor Messenger Ribnucleic Acid in Pituitary Tissue of Orchidectomized Sheep: Effect of Estradiol and Gnrh." Biology of Reproduction **54**: 407-412.

Adler B and Crowley W (1986). "Evidence for Gamma-Aminobutyric Acid Modulation of Ovarian Hormonal Effects on Luteinizing Hormone Secretion and Hypothalamic Catecholamine Activity in the Female Rat." Endocrinology **118**: 91-97.

Alarid ET and Mellon PL (1995). "Down-Regulation of the Gonadotropin-Releasing Hormone Receptor Messenger Ribonucleic Acid by Activation of Adenylyl Cyclase in Alphat3-1 Pituitary Gonadotrope Cells." Endocrinology **136**: 1361-1366.

Albarracin CT, Kaiser UB and Chin WW (1994). "Isolation and Characterization of the 5'-Flanking Region of the Mouse Gonadotropin-Releasing Hormone Receptor Gene." Endocrinology **135**: 2300-2306.

Alexander SL and Irvine CHG (1987). "Secretion Rates and Short-Term Patterns of Gonadotrophin-Releasing Hormone, Fsh, Lh Throughout the Periovulatory Period in the Mare." Journal of Endocrinology **114**: 351-362.

Allen WR, Sanderson MW, Greenwood RES, Ellis DR, Crowhurst JS, Simpson DJ and Rossdale PD (1987). "Induction of Ovulation in Anoestrous Mares with a Slow-Release Implant of a Gnrh Analogue (Ici 118 630)." J Reprod Fertil Suppl **35**: 469-478.

Amoss MR, Blackwell RB, Fellows VR and Guillemin R (1971). "Purification, Amino Acid Composition and N-Terminus of the Hypothalamic Luteinizing Hormone Releasing Factor (Lrf) of Ovine Origin." Biochem Biophys Res Commun **44**: 205-210.

Anderson L, McGregor A, Cook JV, Chilvers E and Eidne KA (1995). "Rapid Desensitization of Gnrh-Stimulated Intracellular Signalling Events in Alpha T3-1 and Hek-293 Cells Expressing the Gnrh Receptor." Endocrinology **136**(11): 5228-5231.

Anderson L, Milligan G and Eidne KA (1993). "Characterization of the Gonadotropin-Releasing Hormone Receptors in Alphat3-1 Pituitary Gonadotroph Cells." J Endocrinology **136**: 51-58.

Arora KK, Sakai A and Catt KJ (1995). "Effects of Second Intracellular Loop Mutations on Signal Transduction and Internalization of the Gonadotropin-Releasing Hormone Receptor." Journal of Biological Chemistry **270**: 22820-22826.

Bakker J and Baum MJ (2000). "Neuroendocrine Regulation of Gnrh Release in Induced Ovulators." Frontiers in Neuroendocrinology **21**: 220-262.

Bauer-Dentoin AC, Weiss J and Jameson JL (1995). "Roles of Estrogen, Progesterone, and Gonadotropin-Releasing Hormone (Gnrh) in the Control of Pituitary Gnrh Receptor Gene Expression at the Time of the Preovulatory Gonadotropin Surges." Endocrinology **136**: 1014-1019.

Beggs MJ and Miller WL (1989). "Gonadotropin-Releasing Hormone-Stimulated Luteinizing Hormone (Lh) Release from Ovine Gonadotrophs in Culture Is Separate from Phorbol Ester-Stimulated Lh Release." Endocrinology **124**(2): 667-674.

Belchetz PE, Plant TM, Nakai Y, Keogh EG and Knobil E (1978). "Hypophyseal Responses to Continuous and Intermittent Delivery of Hypothalamic Gonadotropin-Releasing Hormone." Science **202**: 631-633.

Ben-Menahem D, Shraga-Levine Z, Limor R and Naor Z (1994). "Arachadonic Acid and Lipoxygenase Products Stimulate Gonadotropin Alpha-Subunit Mrna Levels in Pituitary Alphat3-1 Cell Line: Role in Gonadotropin Releasing Hormone Action." Biochemistry **33**: 12795-12799.

Blomenrohr M, Heding A, Sellar R, Leurs R, Bogerd J, Eidne KA and Willars GB (1999). "Pivotal Role for the Cytoplasmic Carboxyl-Terminal Tail of a Nonmammalian Gonadotropin-Releasing Hormone Receptor in Cell Surface Expression, Ligand Binding, and Receptor Phosphorylation and Internalization." Mol Pharmcacol **56**(6): 1229-1237.

Bodner M, Castrillo JL, Theill LE, Deemick T, Ellisman M and Karin M (1988). "The Pituitary-Specific Transcription Factor Ghf-1 Is a Homeobox-Containing Protein." Cell **55**: 505-518.

Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ and McNeilly AS (1993). "Cloning and Sequencing of the Sheep Pituitary Gonadotropin-Releasing Hormone Receptor and Changes in Expression of Its Mrna During the Estrous Cycle." Mol Cell Endocrinol **94**(2): R23-7.

Campion CE, Turzillo AM and Clay CM (1996). "The Gene Encoding the Ovine Gonadotropin-Releasing Hormone (Gnrh) Receptor: Cloning and Initial Characterization." Gene **170**(2): 277-80.

Caraty A, Locatelli A, Delalen B, Spitz M, Schatz B and Bouchard P (1990). "Gonadotropin-Releasing Hormone (Gnrh) Agonists and Gnrh Antagonists Do Not Alter Endogenous Gnrh Secretion in Short-Term Castrated Rams." Endocrinology **127**: 2523-2529.

Carmel PW, Araki S and Ferin M (1976). "Pituitary Stalk Blood Collection in Rhesus Monkeys. Evidence for Pulsatile Release of Gonadotropin Releasing Hormone (Gnrh)." Endocrinology **99**: 243-248.

Cassina MP, Grantham KD and JD Neill (1997). "A Temporally Intermediate Mode of Gonadotropin Releasing Hormone-Induced Desensitization of Luteinizing Hormone Secretion." Molecular and Cellular Endocrinology **132**: 53-59.

Catt KJ, Loumaye E and Katikineni M (1983). Receptors and Actions of Gonadotropin Releasing Hormone (Gnrh) on Pituitary Gonadotrophs. Role of Peptides and Proteins in Control of Reproduction. M. S. a. D. DS. New York, Elsevier: 33-62.

Cermark DL, Braden T, Manns J, Niswender GD and Nett TM (1983). Contents of Hypothalamic Gnrh, Pituitary Fsh and Lh, and Pituitary Receptors for Gnrh and Estradiol in Postpartum Suckled Beef Cows. American Society of Animal Science.
332

Cesnajaj M, Catt KJ and Stojilkovic SS (1994). "Coordinate Action of Calcium and Protein Kinase C in the Expression of Primary Response Gene in Pituitary Gonadotropes." Endocrinology **135**: 692-701.

Chakraborty PK, Reeves JJ and Arimura A (1973). "Serum Lh Levels in Pre-Pubertal Female Pigs Chemically Treated with Synthetic Luteinizing Hormone-Releasing Hormone/Follicle-Stimulating Hormone-Releasing Hormone." Endocrinology **92**: 55-61.

Chang JP, Morgan RO and Catt KJ (1988). "Dependence of Secretory Responses to Gonadotropin-Releasing Hormone on Diacylglycerol Metabolism." J Biol Chem **263**: 18614-18620.

Chauvin S, Berault A, Lerrant Y, Hilbert M and Counis R (2000). "Functional Importance of Transmembrane Helix 6 Trp279 and Exoloop 3 Val299 of Rat Gonadotropin-Releasing Hormone Receptor." Molecular Pharmacology **57**: 625-633.

Cheng KW, Ngan ES, Kang SK, Chow BK and Leung PC (2000). "Transcriptional Down-Regulation of Human Gonadotropin-Releasing Hormone (Gnrh) Receptor Gene by Gnrh: Role of Protein Kinase C and Activating Protein 1 [in Process Citation]." Endocrinology **141**(10): 3611-22.

Cheon M, Park D, Kim K, Park SD and Ryu K (1999). "Homolgous Upregulation of Gnrh Receptor Mrna by Continuous Gnrh in Cultured Rat Pituitary Cells." Endocrine **11**(1): 49-55.

Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP and Sealfon SC (1993). "Cloning and Characterization of the Human Gnrh Receptor." Mol Cell Endocrinol **94**: R1-R6.

Chung H-Y, Yang Q, Catt KJ and Arora KK (1999). "Expression and Function of the Gonadotropin-Releasing Hormone Receptor Are Dependent on a Conserved Apolar Amino Acid in the Third Intracellular Loop." The Journal of Biological Chemistry **274**(50): 35756-35762.

Clarke IJ and Cummins JT (1982). "The Temporal Relationship between Gonadotropin Releasing Hormone (Gnrh) and Luteinizing Hormone (Lh) Secretion in Ovariectomized Ewes." Endocrinology **11**: 1737-1739.

Clarke IJ, Cummins JT, Findlay JK, Burman KJ and Doughton BW (1984). "Effects on Plasma Luteinizing Hormone and Follicle Stimulating Hormone of Varying the Frequency and Amplitude of Gonadotropin-Releasing Hormone Pulses in Ovariectomized Ewes with Hypothalamo-Pituitary Disconnection." Neuroendocrinology **39**: 214-221.

Clay CM, Nelson SE, DiGregorio GB, Campion CE, Wiedemann AL and Nett RJ (1995). "Cell-Specific Expression of the Mouse Gonadotropin-Releasing Hormone (Gnrh) Receptor Gene Is Conferred by Elements Residing within 500 Bp of Proximal 5' Flanking Region." Endocrine **3**: 615-622.

Clayton RN and Catt KJ (1981). "Regulation of Pituitary Gonadotropin-Releasing Hormone Receptors by Gonadal Hormones." Endocrinology **108**: 887-895.

Clayton RN, Solano AR, Garcia-Vela A, Dufau ML and Catt KJ (1980). "Regulation of Pituitary Receptors for Gonadotropin-Releasing Hormone During the Rat Estrous Cycle." Endocrinology **107**: 699-706.

Conn PM, Ganong BR, Ebeling J, Staley D, Niedel J and Bell RM (1985). "Diacylglycerols Release Lh: Structure-Activity Relations and Protein Kinase C." Biochem Biophys Res Commun **126**: 532-539.

Conn PM and Hazum E (1981). "Luteinizing Hormone Release and Gonadotropin-Releasing Hormone (Gnrh) Receptor Internalization: Independent Actions of Gnrh." Endocrinology **109**(6): 2040-2045.

Conn PM, Rogers DC and Sandhu FS (1979). "Alteration of the Intracellular Calcium Level Stimulates Gonadotropin Release from Cultured Rat Anterior Pituitary Cells." Endocrinology **105**(5): 1122-1127.

Conn PM, Rogers DC and Seay SG (1984). "Biphasic Regulation of the Gonadotropin-Releasing Hormone Receptor by Receptor Microaggregation and Intracellular Calcium Levels." Mol Pharmcacol **25**(51-55).

Conn PM, Rogers DC and Sheffield T (1981). "Inhibition of Gonadotropin-Releasing Hormone-Stimulated Luteinizing Hormone Release by Pimozide, Evidence for a Site of Action after Calcium Mobilization." Endocrinology **109**: 1122.

Cook JV and Eidne KA (1997). "An Intramolecular Disulfide Bond between Conserved Extracellular Cystines in the Gonadotropin-Releasing Hormone Receptor Is Essential for Binding and Activation." Endocrinology **138**(7): 2800-2806.

Cook JV, Faccenada E, Anderson L, Couper GC, Eidne KA and Taylor PL (1993). "Effects of Asn87 and Asp318 Mutations on Ligand Binding and Signal Transduction in the Rat Gnrh Receptor." J Endocrinology **139**: R1-R4.

Cornea A, Janovick JA, Lin X and Conn PM (1999). "Simultaneous and Independent Visualization of the Gonadotropin-Releasing Hormone Receptor and Its Ligand: Evidence for Independent Processing and Recycling in Living Cells." Endocrinology **140**(9): 4772-4780.

Cotecchia S, Exum S, Candon MG and Lefkowitz RJ (1990). "Regions of the Alpha$_1$-Adrenergic Receptor Involved in Coupling to Phosphatidylinositol Hydrolysis and Enhanced Sensitivity of Biological Function." Proc. Natl. Acad. Sci. **87**: 2896-2900.

Cowley MA, Rao A, Wright PJ, Illing N, Millar RP and Clarke IJ (1998). "Evidence for Differential Regulation of Multiple Transcripts of the Gonadotropin Releasing Hormone Receptor in the Ovine Pituitary Gland; Effect of Estrogen." Molecular and Cellular Endocrinology **146**: 141-149.

Crespo P, Xu N, Simonds WF and Gutkind JS (1994). "Ras-Dependent Activation of Map Kinase Pathway Mediated by G-Protein Betagamma Subunits." Nature **369**: 418-420.

Crowder ME, Herring RD and Nett TM (1986). "Rapid Recovery of Gonadotroph Function after Down-Regulation of Receptors for Gnrh in Ewes." J Reprod Fertil **78**: 577-585.

Culler MD and Negro Vilar A (1986). "Evidence That Pulsatile Follicle-Stimulating Hormone Secretion Is Independent of Endogenous Luteinizing Hormone-Releasing Hormone." Endocrinology **118**: 609-612.

Dan-Cohen H, Sofer Y, Schwartzmann ML, Natarajan RD, Nadler JL and Naor Z (1992). "Gnrh Activates the Lipooxygenase Pathway in Cultured Pituitary Cells: Role in Gonadotropin Secretion and Evidence for a Novel Autocrine/Paracrine Loop." Biochemistry **31**: 5442-5448.

Davidson JS, Flanagan CA, Becker II, Illing N, Sealfon SC and Millar RP (1994). "Molecular Function of the Gonadotropin-Releasing Hormone Receptor: Insights from Site-Directed Mutagenesis." Molecular and Cellular Endocrinology **100**: 9-14.

DePaolo LV (1988). "Attenuation of Preovulatory Gonadotropin Surges by Epostane: A New Inhibitor of 3,B-Hydroxy-Steroid Dehydrogenase." J Endocrinology **118**: 59-68.

Dieschke DJ, Bhattacharya AN, Atkinson LE and Knobil E (1970). "Circhoral Oscillation of Plasma Lh Levels in the Ovariectomized Rhesus Monkey." Endocrinology **87**: 850-853.

Dohlman HG, Thorner J, Caron MG and Lefkowitz RJ (1991). "Model Systems for the Study of Seven-Transmembrane Segment Receptors." Annnual Review of Biochemistry **60**: 653-688.

Domanski E, Polkowska J and Dubois MP (1980). "Immunocytochemistry of Luteinizing Hormone Releasing Hormone (Lhrh) in the Sheep Hypothalamus During Various Reproductive Stages: Correlation with the Gonadotropic Hormones of the Pituitary." Cell Tissue Research **208**(2): 329-341.

Domanski E, Przekop F, Skubiskewski B and Woliska E (1975). "The Effect of Site of Action of Indoleamines on the Hypothalamic Centers Involved in the Control of Lh Release and Ovulation in Sheep." Neuroendocrinology **17**: 165-273.

Dong KW, Yu KL and Roberts JL (1993). "Identification of a Major up-Stream Transcription Start Site for the Human Progonadotropin-Releasing Hormone Gene Used in Reproductive Tissues and Cell Lines." Molecular endocrinology **7**: 1654-1666.

Duval DL, Ellsworth BS and Clay CM (1999). "Is Gonadotrope Expression of the Gonadotropin Releasing Hormone Receptor Gene Mediated by Autocrine/Paracrine Stimulation of an Activin Response Element?" Endocrinology **140**(4): 1949-1952.

Duval DL, Nelson SE and Clay CM (1997). "The Tripartite Basal Enhancer of the Gonadotropin-Releasing Hormone (Gnrh) Receptor Gene Promoter Regulates Cell-Specific Expression through a Novel Gnrh Receptor Activating Sequence." Molecular endocrinology **11**: 1814-1821.

Eidne KA, Flanagan CA, Harris NS and Millar RP (1987). "Gonadotropin-Releasing Hormone (Gnrh)-Binding Sites in Human Breast Cancer Cell Lines and Inhibitory Effects of Gnrh Antagonist." J. Clin. Endocr. Metab. **64**: 425-432.

Eidne KA, Sellar RE, Couper G, Anderson L and Taylor PL (1992). "Molecular Cloning and Characterisation of the Rat Pituitary Gonadotropin-Releasing Hormone (Gnrh) Receptor." Mol Cell Endocrinol **90**(1): R5-9.

Emons G, Hoffman BC, Ortmann O, Sturm R, Ball P and Knuppen R (1988). "Modulation of Gonadotropin Releasing Hormone Receptor Concentration in Cultured Female Rat Pituitary Cells by Estradiol Treatment." J Steroid Biochem **31**: 751-756.

Evans JW, Hughs JP, Neely DP, Stabenfeldt GH and Winget CM (1979). "Episodic Lh Secretion Patterns in the Mare During the Oestrous Cycle." J Reprod Fertil **27**: 143-150.

Evans MJ and Irvine CHG (1975). "Serum Concentrations of Fsh, Lh, and Progesterone During the Oestrous Cycle and Early Pregnancy in a Mare." J Reprod Fertil **23**: 193-200.

Fan NC, Jeung C, Peng C, Ofofsson J, Krisinger J and Leung PCK (1994). "The Human Gonadotropin-Releasing Hormone (Gnrh) Receptor Gene: Cloning, Genomic Organization and Chromosomal Assignment." Mol Cell Endocrinol **103**: R1-R6.

Fan NC, Peng C, Krisinger J and Leung PCK (1995). "The Human Gonadotropin-Releasing Hormone Receptor Gene: Complete Structur Including Multiple Promoters, Transcription Initiation Sites, and Polyadenylation Signals." Mol Cell Endocrinol **107**: R1-R8.

Ferguson SS, Downey WE, Calpietro AM, Barak LS, Menard L and Caron MG (1996). "Role of Beta-Arrestin in Mediating Agonist-Promoted G Protein-Coupled Receptor Internalization." Science **271**: 363-366.

Ferguson SS, Menard L, Barak LS, Koch WJ, Colapietro A-M and Caron MG (1995). "Role of Phosphorylation in Agonist-Promoted Beta 2-Adrenergic Receptor Sequestration." J Biol Chem **270**: 24782-24789.

Fernandez-Vasquez G, Kaiser UB, Albarracin CT and Chin WW (1996). "Transcriptional Activation of the Gonadotropin-Releasing Hormone Receptor Gene by Activin A." Molecular endocrinology **10**: 356-366.

Flanagan CA, Millar RP and Illing N (1997). "Advances in Understanding Gonadotropin-Releasing Hormone Receptor Structure and Ligand Interactions." Reviews of Reproduction **2**: 113-120.

Flugge G, Oertel WH and Wuttke W (1986). "Evidence for Estrogen-Receptive Gabaergic Neurons in the Preoptic/Anterior Hypothalamic Area of the Rat Brain." Neuroendocrinology **43**: 1-5.

Fukushima Y, Asano T, Takata K, Funaki M, Oginara T, Anai M, Tsukuda K, Saitoh T, Katagiri H, Aihara M, Matsuhashi N, Oka Y, Yazak Y and Sugano K (1997). "Role of the C Terminus in Histamine H_2 Receptor Signaling, Desensitization, and Agonist-Induced Internalization." J Biol Chem **272**: 19464-19470.

Garcia MC and Ginther OJ (1975). "Plasma Luteinizing Hormone Concentrations in Mares Treated with Gonadotropin-Releasing Hormone and Estradiol." Am J Vet Res **36**: 1581-1584.

Garcia MC and Ginther OJ (1976). "Effects of Ovariectomy and Season on Plasma Luteinizing Hormones in Mares." Endocrinology **98**: 958-962.

Garcia MC and Ginther OJ (1978). "Regulation of Plasma Lh by Estradiol and Progesterone in Ovariectomized Mares." Biology of Reproduction **19**: 447-453.

Gilman AG (1989). "G Proteins and Regulation of Adenylyl Cyclase." JAMA **262**(13): 1819-1825.

Goodman OB Jr, Krupnick JG, Santini F, Gurevick VV, Penn RB, Gagnon AW, Keen JH and Benovic JL (1996). "Beta-Arrestin Acts as a Clathrin Adaptor in Endocytosis of the Beta2-Adrenergic Receptor." Nature **383**(6599): 447-450.

Gorospe WC and Conn PM (1988). "Restoration of the Lh Secretory Response in Desensitized Gonadotropes." Mol Cell Endocrinol **59**: 101-110.

Greaves H, Porter MB and Sharp DC (2000). "Effects of Estradiol Administration on Lh Secretion and Pituitary Responsiveness to Gnrh in Long Term Ovariectomized Pony Mares." J Reprod Fertil(In press).

Gregg DW, Allen MC and Nett TM (1990). "Estradiol-Induced Increase in Number of Gonadotropin-Releasing Hormone Receptors in Cultured Ovine Pituitary Cells." Biology of Reproduction **43**: 1032-1036.

Gregg DW, Schwall RH and Nett TM (1991). "Regulation of Gonadotropin Secretion and Number of Gonadotropin-Releasing Hormone Receptors by Inhibin, Activin-a, and Estradiol." Biology of Reproduction **44**: 725-732.

Grosse R, Schmid A, Schoneberg T, Herrlich A, Muhn P, Schultz G and Gudermann T (2000). "Gonadotropin-Releasing Hormone Receptor Initiates Multiple Signaling Pathways by Exclusively Coupling to Gq/11 Proteins." The Journal of Biological Chemistry **275**(13): 9193-9200.

Grubaugh WR and Sharp DC (1987). "Use of Push-Pull Perfusion Technique in Studies of Gonadotropin-Releasing Hormone Secretion in Mares." J Reprod Fertil Suppl **35**: 289-96.

Haisenleder DJ, Cox ME, Parsons SJ and Marshall JC (1998). "Gonadotropin-Releasing Hormone Pulses Are Required to Maintain Activation of Mitogen-Activated Protein Kinase: Role in Stimulation of Gonadotrope Gene Expression." Endocrinology **139**(7): 3104-3111.

Haisenleder DJ, Yasin M and Marshall JC (1997). "Gonadotropin Subunit and Gonadotropin-Releasing Hormone Receptor Gene Expression Are Regulated by Alterations in the Frequency of Calcium Pulsatile Signals." Endocrinology **138**(12): 5227-30.

Han XB and Conn PM (1999). "The Role of Protein Kinases a and C Pathways in the Regulation of Mitogen-Activated Protein Kinase Activation in Response to Gonadotropin-Releasing Hormone Receptor Activation." Endocrinology **140**(5): 2241-2251.

Hart PJ, Squires EL, Imel KJ and Nett TM (1984). "Seasonal Variation in Hypothalamic Content of Gonadotropin-Releasing Hormone (Gnrh), Pituitary for Gnrh, and Pituitary Content of Luteinizing Hormone and Follicle-Stimulating Hormone in the Mare." Biology of Reproduction **30**: 1055-1062.

Hashizume T, Wei-Hsiung Y, Clay CM and Nett TM (2001). "Internalization Rates of Murine and Ovine Gonadotropin-Releasing Hormone Receptors." Biology of Reproduction: 898-903.

Hawes BE, Barnes S and Conn PM (1993). "Cholera Toxin and Pertussis Toxin Provoke Differential Effects on Luteinizing Hormone Release, Inositol Phosphate Production, and Gonadotropin Releasing Hormone Receptor Binding in the Gonadotrope: Evidence for Multiple Guanyl Nucleotide Binidng Proteins in Gnrh Action." Endocrinology **132**: 2124-2130.

Hawes BE, Waters SB, Janovick JA, Bleasdale JE and Conn PM (1992). "Gonadotropin-Releasing Hormone-Stimulated Intracellular Ca2+ Fluctuations and Luteinizing Hormone Release Can Be Uncoupled from Inositol Phosphate Production." Endocrinology **130**: 3475-3483.

Hazum E, Cuatrecasas P, Marian J and Conn PM (1980). "Receptor-Mediated Internalization of Fluorescent Gonadotropin-Releasing Hormone by Pituitary Gonadotropes." Proc. Natl. Acad. Sci. **77**: 6692-6695.

Heding A, Vrecl M, Bogerd J, McGregor A, Sellar R, Taylor PL and Eidne KA (1998). "Gonadotropin-Releasing Hormone Receptors with Intracellular Carboxyl-Terminal Tails Undergo Acute Desensitization of Total Inositol Phosphate Production and Exhibit Accelerated Internalization Kinetics." The Journal of Biological Chemistry **273**(19): 11472-11477.

Heding A, Vrecl M, Hanyaloglu AC, Sellar R, Taylor PL and Eidne KA (2000). "The Rat Gonadotropin-Releasing Hormone Receptor Internalizes Via a Beta-Arrestin-Independent, but Dynamin-Dependent, Pathway: Addition of a Carboxyl-Terminal Tail Confers Beta-Arrestin Dependency." Endocrinology **114**(1): 299-306.

Horn F, Bilezikjian LM, Perrin MH, Bosma MM, Windle JJ, Huber KS, Blount AL, Hille B, Vale W and Mellon PL (1991). "Intracellular Responses to Gonadotropin-Releasing Hormone in a Clonal Cell Line of the Gonadotrope Lineage." Molecular endocrinology **5**: 347-355.

Hsieh KP and Martin TFJ (1992). "Thyrotropin-Releasing Hormone and Gonadotropin-Releasing Hormone Receptors Activate Phospholipase C by Coupling to the Guanosine-Triphosphate-Binding Proteins Gq and G11." Molecular endocrinology **6**: 1673-1681.

Huckle WR, Hawes BE and Conn PM (1989). "Protein Kinase C-Mediated Gonadotropin-Releasing Hormone Receptor Sequestration Is Associated with Uncoupling of Phosphoinositide Hydrolysis." The Journal of Biological Chemistry **264**(15): 8619-8626.

Hughs JP, Stebenfeldt GH and Evans MJ (1972). "Estrous Cycle and Ovulation in the Mare." J Am Vet Med Assoc **161**: 1367-1374.

Illing N, Jacobs GF, Becker II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealfon SC and Millar RP (1993). "Comparative Sequence Analysis and Functional Characterization of the Cloned Sheep Gonadotropin-Releasing Hormone Receptor Reveal Differences in Primary Structure and Ligand Specificity among Mammalian Receptors." Biochem Biophys Res Commun **196**(2): 745-51.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE and Millar RP (1999). "Two Gonadotropin-Releasing Hormone Receptor Subtypes with Distinct Ligand Selectivity and Differential Distribution in Brain and Pituitary in the Goldfish (Carassius Auratus)." Proc. Natl. Acad. Sci. **96**: 2526-2531.

Imagawa M, Chiu R and Karin M (1987). "Transcription Factor Ap-2 Mediates Induction by Two Different Signal-Transduction Pathways: Protein Kinase C and Camp." Cell **51**: 251-260.

Ingraham HA, Flynn SE, Voss JW, Albert VR, Kapiloff MS, Wilson L and Rosenfeld MG (1990). "The Pou-Specific Domain of Pit-1 Is Essential for Sequence-Specific High Affinity DNA Binding and DNA-Dependent Pit-1-Pit-1 Interactions." Cell **61**: 1021-1033.

Innamorati G, Sadeghi H and Birnbaumer (1999). "Phosphorylation and Recycling Kinetics of G Protein-Coupled Receptors." J Recept Signal Tranduct Res **19**(1-4): 315-326.

Irvine CHG (1995). "The Nonpregnancy Mare: A Review of Some Current Research and of the Last 25 Years of Endocrinology." Biology of Reproduction Monolog **1**: 343-360.

Irvine CHG and Alexander SL (1994b). "The Dynamics of Gonadotropin-Releasing Hormone, Lh, and Fsh Secretion During the Spontaneous Ovulatory Surge of the Mare as Revealed by Intensive Sampling of Pituitary Venous Blood." J Endocrinology **140**: 283-295.

Janovick JA and Conn PM (1996). "Gonadotropin Releasing Hormone Agonist Provokes Homologous Receptor Microaggregation: An Early Event in Seven-Transmembrane Receptor Mediated Signaling." Endocrinology **137**(8): 3602-3605.

Janovick JA, Natarajan K, Longo F and Conn PM (1991). "Caldesmon: A Bifunctional (Calmodulin and Actin) Binding Protein Which Regulates Stimulated Gonadotropin Release." Endocrinology **1991**(129).

Jarjeur L, Jandelsman D, Raum W and Swerdloff R (1986). "Mechanism of Action of Dopamine on the in Vitro Release of Gonadotropin-Releasing Hormone." Endocrinology **119**: 1726-1732.

Jennes L, Beckman WC, Stumph WE and Grzanna R (1982). "Anatomical Relationships of Sertonergic and Noradrenalinerigic Projections with the Gnrh System in Septum and Hypothalamus." Exp Brain Res **46**: 331-338.

Jennes L, Bronson D, Stumpf WE and Conn PM (1985). "Evidence for an Association between Calmodulin and Membrane Patches Containing Gonadotropin-Releasing Hormone Receptor Complexes in Cultured Gonadotropes." Cell Tissue Research **239**: 311.

Kaiser UB, Jakubowiak A, Steinberger A and Chin WW (1993). "Regulation of Rat Pituitary Gonadotropin-Releasing Hormone Receptor Mrna Levels in Vivo and in Vitro." Endocrinology **133**(2): 931-934.

Kaiser UB, Zhao D, Cardona GR and Chin WW (1992). "Isolation and Characterization of Cdnas Encoding the Rat Pituitary Gonadotropin-Releasing Hormone Receptor." Biochem Biophys Res Commun **189**(3): 1645-52.

Kakar SS (1997). "Molecular Structure of the Human Gonadotropin-Releasing Hormone Receptor Gene." European Journal of Endocrinology **137**: 183-192.

Kakar SS, Grantham K, Musgrove LC, Devor D, Sellers JC and Neill JD (1994a). "Rat Gonadotropin-Releasing Hormone (Gnrh) Receptor: Tissue Expression and Hormonal Regulation of It Mrna." Molecular and Cellular Endocrinology **101**: 151-157.

Kakar SS, Grizzle WF and Neill JD (1994b). "The Nucleotide Sequence of Human Gnrh Receptors in Breast and Ovarian Tumors Are Identical with That Found in Pituitary." Mol Cell Endocrinol **106**: 145-149.

Kakar SS and Jennes L (1995). "Expression of Gonadotropin-Releasing Hormone and Gonadotropin-Releasing Hormone Receptor Mrnas in Various Non-Reproductive Tissues." Cancer Letters **98**: 57-62.

Kakar SS, LC Musgrove, DC Devor, Sellers and JD Neill (1992). "Cloning, Sequencing, and Expression of Human Gonadotropin Releasing Hormone (Gnrh) Receptor." Biochemical and Biophysical Research Communications **189**: 289-295.

Kakar SS, Rahe CH and Neill JD (1993a). "Molecular Cloning, Sequencing, and Characterizing the Bovine Receptor for Gonadotropin Releasing Hormone (Gnrh)." Domest Anim Endocrinol **10**(4): 335-42.

Kakar SS, Rahe CH and Neill JD (1993b). "Molecular Cloning, Sequencing and Characterizing the Bovine Receptor for Gonadotropin Releasing Hormone (Gnrh)." Domest Anim Endocrinol **10**: 335-342.

Kalra PS and McCann SM (1975). "The Stimulatory Effect of Gonadotropin Release of Implants of Estradiol or Progesterone in Certain Sites in the Central Nervous System." Neuroendocrinology **19**: 289-302.

Kalra SP and Kalra PS (1974). "Temporal Interrelationships between Circulating Levels of Estradiol, Progesterone and Lh During the Rat Estrous Cycle: Effects of Exogenous Progesterone." Endocrinology **95**: 1711-1718.

Kang SK, Cheng KW, Ngan ESW, Chow BKC, Choi KC and Leung PCK (2000). "Differential Expression of Human Gonadotropin-Releasing Hormone Receptor Gene in Pituitary and Ovarian Cells." Molecular and Cellular Endocrinology **162**: 157-166.

Karsch FJ, Cummins JT, Thomas GB and Clark IJ (1987). "Steroid Feedback Inhibition of Pulsatile Secretion of Gonadotropin Releasing Hormone in the Ewe." Biology of Reproduction **36**: 1207-1218.

Karsch FJ, Foster DL, Bittman EL and Goodman RL (1983). "A Role for Estradiol in Enhancing Luteinizing Hormone Pulse Frequency During the Follicular Phase of the Estrous Cycle of Sheep." Endocrinology **113**: 1333-1339.

Karsch FJ, Legan SL, Hauger RL and Foster DL (1977). "Negative Feedback Action of Progesterone on Tonic Luteinizing Hormone Secretion in the Ewe: Dependence on the Ovaries." Endocrinology **101**: 800-808.

Katt JA, Duncan JA, Herbon L, Barkan A and Marshall JC (1985). "The Frequency of Gonadotropin-Releasing Hormone Stimulation Determines the Number of Pituitary Gonadotropin-Releasing Hormone Receptors." Endocrinology **116**: 2113-2115.

Kaynard AH, Malpaux B, Robinson JE, Wayne NL and Karsch FJ (1988). "Importance of Pituitary and Neural Actions of Estradiol in Induction of the Lh Surge in the Ewe." Neuroendocrinology **48**: 296-303.

Keinan D and Hazum E (1985). "Mapping of Gonadotropin-Releasing Hormone Receptor Binding Site." Biochemistry **24**: 7728-7732.

Kesner JS (1988). "Site of Action for the Estradiol-Induced Luteinizing Hormone Surge in Farm Animals and Primates." Domest Anim Endocrinol **5**: 265-281.

Kirkpatrick BL, Esquivel E, Gentry PC, Moss GE, Wise ME and Hamernik DL (1998). "Regulation of Amounts of Mrna for Gnrh Receptors by Estradiol and Progesterone in Sheep." Endocrine **8**(1): 98-99.

Knobil E (1981). "Patterns of Hypophysiotropic Signals and Gonadotropin Secretion in the Rhesus Monkey." Biology of Reproduction **24**: 44-49.

Koenig JA and Edwardson JM (1997). "Endocytosis and Recycling of G Protein-Coupled Receptors." Trends Pharmacol Sci **18**: 276-287.

Koiter TR, Pols-Valkhof N, Zurcher AF and Schuiling GA (1979). "Lh-Secretory Response Caused by Continuous Infusion of Lh-Rh in Pseudopregnant Rats." Acta Endocrinol **92**: 28-45.

Kottler ML, Bergametti F, Carre M-C, Morice S, Decoret E, Lagarde J-P, Starzec A and Counis R (1999). "Tissue-Specific Pattern of Variant Transcripts of the Human Gonadotropin-Releasing Hormone Receptor Gene." European Journal of Endocrinology **140**: 561-569.

L Chi, A Prikhozhan, C Flanagan, JS Davidson, M Golembo, N Illing, RP Millar and SC Sealfon (1993). "Cloning and Characterization of the Human Gnrh Receptor." Molecular and Cellular Endocrinology **91**: R1-R6.

Laws SC, Webster JC and Miller WL (1990). "Estradiol Alters the Effectiveness of Gonadotropin-Releasing Hormone (Gnrh) in Ovine Pituitary Cultures: Gnrh Receptors Vs. Responsiveness to Gnrh." Endocrinology **127**: 381-386.

Lebhsetwar AP (1972). "Role of Monoamines in Ovulation: Evidence for a Serotonergic Pathway for Inhibition of Spontaneous Ovulation." J Endocrinology **54**: 269-275.

Lefkowitz RJ (1998). "G Protein-Coupled Receptors: Iii. New Roles for Receptor Kinases and Beta-Arrestins in Receptor Signaling and Desensitization." J Biol Chem **273**: 18677-18680.

Levine JE, Pay K-YF, Ramirez BD and Jackson GL (1982). "Simultaneous Measurement of Luteinizing Hormone-Releasing and Luteinizing Hormone Release in Unanaesthetized Ovariectomized Sheep." Endocrinology **111**: 1449-1455.

Levine JE and Ramirez VD (1980). "In Vivo Release of Luteinizing Hormone-Releasing Hormone Estimated with Push-Pull Cannulae from the Mediobasal Hypothalami of Ovariectomized,Steroid-Primed Rats." Endocrinology **107**: 1782-1791.

Liggett SB, Ostrowski J, Chesnut LC, Kuruse H, Raymond JR, Caron MG and Lefkowitz RJ (1992). "Sites in the Third Intracellular Loop of the A2a-Adrenergic Receptor Confer Short Term Agonist-Promoted Desensitization. Evidence for a Receptor Kinase-Mediated Hormone." J Biol Chem **267**: 4740-4746.

Lin X, Janovick JA, Brothers S, Blomenrohr M, Bogerd J and Conn M (1998a). "Addition of Catfish Gonadotropin-Releasing Hormone (Gnrh) Receptor Intracellular Carboxyl-Terminal Tail to Rat Gnrh Receptor Alters Receptor Expression and Regulation." Molecular endocrinology **12**: 161-171.

Lin X, Janovick JA and Conn M (1998b). "Mutations at the Consensus Phsophorylation Sites in the Third Intracellular Loop of the Rat Gonadotropin-Releasing Hormone Receptor: Effects of Receptor Ligand Binding and Signal Transduction." Biology of Reproduction **59**: 1470-1476.

Lorenz W, Inglese J, Palczewski K, Onorato JJ, Caron MG and Lefkowitz RJ (1991). "The Receptor Kinase Family: Primary Structure of Rhodopsin Kinase Reveals Similarities to the Beta-Adrenergic Receptor Kinase." Proc. Natl. Acad. Sci. **88**: 8715-8719.

Loumaye E and Catt KJ (1982). "Homologous Regulation of Gonadotropin-Releasing Hormone Receptors in Cultured Pituitary Cells." Science **215**: 983-985.

Marantz Y, Reiss N, Przedecki F and Naor Z (1995). "Involvement of Protein Phosphatases in Gnrh Regulated Gonadotropin Secretion." Mol Cell Endocrinol **111**: 7-11.

Marian J and Conn PM (1979). "Gonadotropin Releasing Hormone Stimulation of Cultured Pituitary Cells Requires Calcium." Mol Pharmcacol **16**: 196.

Marshall JC, Bourne GA, Frager MS and Pieper DR (1983). Pituitary Gonadotropin Releasing Hormone (Gnrh) on Pituitary Gonadotrophs. Role of Peptides and Proteins in Control of Reproduction. M. S. a. D. DS. New York, Elsevier: 33-62.

Mason DR, Arora KK, Mertz LM and Catt KJ (1994). "Homologous Down-Regulation of Gonadotropin-Releasing Hormone Receptor Sites and Messenger Ribonucleic Acid Transcripts in Alpha T3-1 Cells." Endocrinology **135**(3): 1165-70.

Matsuo H, Baba Y, Nair RMG, Arimura A and Shally AV (1971). "Structure of the Porcine Lh and Fsh-Releasing Hormone. I. The Proposed Amino Acid Sequence." Biochem Biophys Res Commun **43**: 1334-1339.

Matteri RL, Roser JF, Baldwin DM, Lipovetsky V and Papkoff H (1987). "Characterization of a Monoclonal Antibody Which Detects Luteinizing Hormone from Diverse Mammalian Species." Domest Anim Endocrinol **4**(3): 157-165.

McArdle CA, Forrest-Owen W, Willars G, Davidson J, Poch A and Kratzmeier M (1995). "Desensitization and Gonadotropin-Releasing Hormone Action in the Gonadotrope-Derived Alphat3-1 Cell Line." Endocrinology **136**: 4864-4871.

McArdle CA, Gorospe WC, Huckle WR and Conn PM (1987). "Homologous Down-Regulation of Gonadotropin-Releasing Hormone Receptors and Desensitization of Gonadotropes: Lack of Dependence on Protein Kinase C." Mol Endocrinol **1**(6): 420-9.

McArdle CA, Schomerus E, Groner I and Poch A (1992). "Estradiol Regulates Gonadotropin-Releasing Hormone Receptor Number, Growth and Inositol Phosphate Production in Alphat3-1 Cells." Mol Cell Endocrinol **87**: 95-103.

McCann SM (1974). "Regulation of Secretion of Follicle Stimulating Hormone and Luteinizing Hormone." Handbook of Physiology **4**: 489-517.

McCann SM, Krulich L and Ojeda SR (1979). Neurotransmitters in Control of Anterior Pituitary Function. F. K. a. H. T. New York, Plenum Press: 227-233.

McEwen BS and Parsons B (1982). "Gonadal Steroid Action on the Brain: Neurochemistry and Neuropharmacology." Annu Rev Pharmacol Toxicol **22**: 555-598.

Melrose PA, Pickel C, Cheramie HS, Henk WG and Littlefied-Chaubaud MA (1994). "Distribution and Morphology of Immunoreactive Gonadotropin-Releasing Hormone Neurons in the Basal Forebrain of Ponies." J Comp Neurol **339**(2): 269-287.

Merchenthaler I, Lopez FJ and Negro-Vilar A (1990). "Co-Localization of Galanin and Luteinizing Hormone-Releasing Hormone in a Subset of Preoptic-Hypothalamic Neurons." Proc Natl Acad Sci U S A **87**: 6326-6330.

Merelli F, Stojilkovic SS, Iida T, Krsmanovic LZ, Zheng L, Mellon PL and Catt KJ (1992). "Gonadotropin-Releasing Hormone-Induced Calcium Signaling in Clonal Pituitary Gonadotrophs." Endocrinology **131**: 925-932.

MH Perrin, LM Bilezekjian, C Hoeger, CJ Donaldson, J Rivier, Y Hass and WW Vale (1993). "Molecular and Functional Characterization of Gnrh Receptors Cloned from Rat Pituitary and Mouse Pituitary Tumor Cell Line." Biochemical and Biophysical Research Communications 191 1139-1144 **191**: 1139-1144.

Millan M, Aguilera G, Wynn PC, Mendelsohn FAO and Catt KJ (1985). "Autoradiographic Localization of Brain Receptors for Peptide Hormones: Angiotensin Ii, Coritocotropin-Releasing Factor, and Gonadotropin-Releasing Hormone." Methods Enzymol **124**: 590-607.

Minaretzis D, Jakubowski M, Mortola JF and Pablou SN (1995). "Gonadotropin-Releasing Hormone Receptor Gene Expression in Human Ovary and Granulosa-Lutein Cells." J. Clin. Endocr. Metab. **80**: 430-434.

Morel G, Dihl F, Aubert ML and Dubois PM (1987). "Binding and Internalization of Native Gonadoliberin (Gnrh) by Anterior Pituitary Gonadotrophs of the Rat. A Quantitative Autoradiographic Study after Cryoultramicrotomy." Cell Tissue Research **248**(3): 541-550.

Morgan RO, Chang JP and Catt KJ (1987). "Novel Aspects of Gonadotropin-Releasing Hormone Activates a Rapid Ca2+ -Indepenedent Phosphodiester Hydrolysis of Polyphosphoinositides in Pituitary Gonadotrophs." J Biol Chem **262**: 1166-1171.

Moss RL and McCann SM (1973). "Induction of Mating Behavior in Rats by Luteinizing Hormone-Releasing Factor." Science **181**: 177-179.

Myburgh DB, Millar RP and Hapgood JP (1998a). "Alanine-261 in Intracellular Loop Iii of the Human Gonadotropin-Releasing Hormone Receptor Is Crucial for G-Protein Coupling and Receptor Internalization." Biochem J **331**: 893-896.

Myburgh DB, Pawson AJ, Davidson JS, Flanagan CA, Millar RP and Hapgood JP (1998b). "A Single Amino Acid Substitution in Transmembrane Helix Vi Results in Overexpression of the Human Gnrh Receptor." European Journal of Endocrinology **139**: 438-447.

Naor I, Benard I and Seger I (2000). "Activation of Mapk Cascades by G-Protein-Coupled Receptors: The Case of Gonadotropin-Releasing Hormone Receptor." Trends Endocrinol Metab **11**(3): 91-99.

Naor Z (1990). "Signal Transduction Mechanisms of Ca2+ Mobilizing Hormones: The Case of Gonadotropin-Releasing Hormone." Endocr Rev **11**: 326-353.

Naor Z (1991). "Is Arachadonic Acid a Second Messenger in Signal Transduction?" Mol Cell Endocrinol **80**: 181-186.

Naor Z, Azard A, Limor R, Zakut H and Lotan M (1986). "Gonadotropin-Releasing Hormone Activates a Rapid Ca2+ -Independent Phosphodiester Hydrolysis of Polyphosphoinositides in Pituitary Gonadotrophs." J Biol Chem **216**: 12506-12512.

Naor Z and Childs GV (1986). "Binding and Activation of Gonadotropin-Releasing Hormone Receptors in Pituitary and Gonadal Cells." Int Rev Cytol **103**: 147-156.

Naor Z, Dan-Hohen H, Hermon J and Limor R (1989). "Induction of Exocytosis in Permeabilized Pituitary Cells by Alpha- and Beta-Type Protein Kinase C." Proc. Natl. Acad. Sci. **88**: 4501-4504.

Naor Z and Eli Y (1985). "Synergistic Stimulation of Luteinizing Hormone (Lh) Release by Protien Kinase C Activators and Ca^{2+} -Ionophore." Biochem Biophys Res Commun **130**(2): 848-
853.

Naor Z, Harris D and Shacham S (1998). "Mechanism of Gnrh Receptor Signaling: Combinatorial Cross-Talk of Ca^{2+} and Protein Kinase C." Frontiers in Neuroendocrinology **19**: 1-19.

Negro-Villar A, Advis J, Ojeda S and McCann S (1982). "Pulsatile Luteinizing Hormone (Lh) Patterns in Ovariectomized Rats: Involvement of Norepinephrine and Dopamine in the Release of Lh-Releasing Hormone and Lh." Endocrinology **111**(932-938).

Nehrenberg WB, Corder R and Gaillard RC (1989). "A Physiological Role for Neuropeptide Y in Regulating the Estrogen/Progesterone Induced Luteinizing Hormone Surge in Ovariectomized Rats." Neuroendocrinology **48**: 680-682.

Neill JD, Duck W, Sellers JC, Musgrove LC, Scheschonka A, Druey KM and Kehrl JH (1997a). "Potential Role for a Regulator of G Protein Signaling (Rgs3) in Gonadotropin-Releasing Hormone (Gnrh) Stimulated Desensitization." Endocrinology **138**(2): 843-846.

Neill JD, Sellers JC, Musgrove LC and Duck LW (1997b). "Epitope-Tagged Gonadotropin-Releasing Hormone Receptors Heterologously-Expressed in Mammalian (Cos-1) and Insect (Sf9) Cells." Mol Cell Endocrinol **127**: 143-154.

Nelson S, Horvat RD, Malvey J, Roess DA, Barisas BG and Clay CM (1999). "Characterization of an Intrinsically Fluorescent Gonadotropin-Releasing Hormone Receptor and Effects of Ligand Binding on Receptor Lateral Diffusion." Endocrinology **140**(2): 950-957.

Nett TM, Crowder ME, Moss GE and Duello TM (1981). "Gnrh-Receptor Interaction. V. Down-Regulation of Pituitary Receptors for Gnrh in Ovariectomized Ewes by Infusion of Homologous Hormone." Biology of Reproduction **24**: 1145-1155.

Nishizuka Y (1992). "Intracellular Signaling by Hydrolysis of Phospholipids and Activation of Protein Kinase C." Science **258**: 607-614.

Norwitz ER, Cardona GR, Kyeong-Hoon J and Chin WW (1999). "Identification and Characterization of the Gonadotropin-Releasing Hormone Response Elements in the Mouse Gonadotropin-Releasing Hormone Receptor Gene." The Journal of Biological Chemistry **275**(2): 867-880.

Orias R, Negro-Vilar A, Libertun C and McCann SM (1974). "Inhibitory Effect on Lh Release of Estradiol Injected into the Third Ventricle." Endocrinology **94**: 852-855.

Palczewski K and Benovic JL (1991). "G-Protein-Coupled Receptor Kinases." Trends in Biochemical Sciences **16**: 387-391.

Park OK (1990). "Gonadotropin-Releasing Hormone Gene Expression During Rat Estrous Cycles: Effects of Pentobarbital and Ovarian Steroids." Endocrinology **127**: 365.

Pawson AK, Katz A, Sun Y-M, Lopes J, Illing N, Millar RP and Davidson JS (1998). "Contrasting Internalization Kinetics of Human and Chicken Gonadotropin-Releasing Hormone Receptors Mediated by C-Terminal Tail." J Endocrinology **156**: R9-R12.

Peng C, Fan NC, Ligier M, Vaananen J and Leung PCK (1994). "Expression and Regulation of Gonadotropin-Releasing Hormone (Gnrh) and Gnrh Receptor Messenger Ribonucleic Acids in Human Granulosa-Luteal Cells." Endocrinology **135**: 1740-1746.

Plotka ED (1975). "Periovulatory Changes in Peripheral Plasma Progesterone and Estrogen Concentrations in the Mare." Am J Vet Res **36**: 1359-1362.

Porter MB, Cleaver BD, Peltier M, Robinson G and Sharp DC (1997a). "The Effect of Pulsatile Gonadotropin-Releasing Hormone and Estradiol Administration in Luteinizing Hormone and Follicle-Stimulating Hormone Concentrations in Pituitary Stalk-Sectioned Ovariectomized Pony Mares." Domest Anim Endocrinol **14**(5): 275-285.

Porter MB, Cleaver BD, Peltier M, Robinson G, Thatcher WW and Sharp DC (1997b). "Comparative Study between Pony Mares and Ewes Evaluating Gonadotrophic Responses to Administration of Gonadotrophin-Releasing Hormone." Journal of Reproduction and Fertility **110**(219-229).

Porter MB, Peltier M, Robinson G, Cleaver BD and Sharp DC (1996). Effect of Acute Estradiol Treatment on the Dynamics of Luteinizing Hormone and Gonadotropin-Releasing Hormone Release in Ovariectomized Pony Mares. Annual Meeting for The Society of the Study of Reproduction, Ann Harbor, Michigan.

Pozor MA, McDonnel SM, Kenney RM and Tischner M (1991). "Gnrh Facilitates Copulatory Behavior in Geldings Treated with Testosterone." J Reprod Fertil Suppl **4**: 666-667.

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG and Cormier MJ (1992). "Primary Structure of the *Aequorea Victoria* Green-Fluorescent Protein." Gene **111**: 229-233.

Probst WC, Snyder LA, Schuster DI, Brosius J and Sealfon SC (1992). "Sequence Alignment of the G-Protein Coupled Receptor Superfamily." DNA Cell Biol. **11**: 1-20.

Przkeop F, Skubiskewski B and Domanski E (1975). "The Effects of Indoleamine (Serotonin and Melatonin) in Induction of Ovulation in Rabbits." Acta Physiol Pol **26**: 395-443.

Ramirez VD and Dluzen DE (1987). "Is Progesterone a Pre-Hormone in Cns?" J Steroid Biochem **27**: 589-598.

Rao IM and Mahesh VB (1986). "Role of Progesterone in the Modulation of the Preovulatory Surge of Gonadotropins and Ovulation in the Pregnant Mare's Serum Gonadotropin-Primed Immature Rat and Adult Rat." Biology of Reproduction **35**: 1154-1161.

Reinhart J and Catt KJ (1995). Structure of the Rat Gnrh Receptor Gene. 77th Annual Meeting of the Endocrine Society, Washington DC.
Abstract No. P1-537

Reinhart J, Mertz LM and Catt KJ (1992a). "Molecular Cloning and Expression of Cdna Encoding the Murine Gonadotropin-Releasing Hormone Receptor." Journal of Biological Chemistry **267**(21): 281-284.

Reinhart J, Mertz LM and Catt KJ (1992b). "Molecular Cloning and Expression of Cdna Encoding the Murine Gonadotropin-Releasing Hormone Receptor." J Biol Chem **267**(30): 21281-21284.

Sabatino FD, Collins P and McDonald JK (1989). "Neuropeptide-Y Stimulation of Luteinizing Hormone-Releasing Hormone Secretion from the Median Eminence in Vitro by Estrogen-Dependent and Extracellular Ca^{2+}-Independent Mechanisms." Endocrinology **124**(5): 2089-2098.

Sakurai H, Adams BM and Adams TE (1997). "Concentration of Gnrh Receptor and Gnrh Receptor Mrna in Pituitary Tissue of Orchidectomized Sheep: Effect of Oestradiol, Progesterone, and Progesterone Withdrawl." J Endocrinology **152**(1): 91-98.

Samli MH and Geschwind II (1968). "Some Effects of Energy Transfer Inhibitors and of Ca^{2+} -Free and K^{+} -Enhanced Media on the Release of Lh from the Rat Pituitary Gland *in Vitro.*" Endocrinology **82**: 225.

Santen RJ and Bardin CW (1973). "Episodic Luteinizing Hormone Secretion in Man: Pulse Analysis, Clinical Interpretation, Physiologic Mechanisms." J Clin Invest **52**: 2617-2628.

Schanbacker BD and Winstra DD (1977). "Acute and Chronic Effects of Gonadotropin-Releasing Hormone on Reproductive Characteristics of Rams During the Nonbreeding Season." J Anim Sci **44**: 650-655.

Sealfon SC, Weinstein H and Millar RP (1997). "Molecular Mechanisms of Ligand Interaction with the Gonadotropin- Releasing Hormone Receptor." Endocr Rev **18**(2): 180-205.

Seger R and Krebs EG (1995). "The Mapk Signaling Cascade." FASEB J **9**: 726-735.

Setalo GS, Vigh AV, Schally A and Flerko B (1975). "Lh-Rh Containing Neural Elements on the Rat Hypothalamus." Endocrinology **96**: 135-142.

Shah BH and Milligan G (1993). "Activation of the Gonadotrophin Releasing Hormone Receptor of Alphat3-1 Cells Results in Downregulation of the Alpha Subunit of Both Gq/G11." Biochem Soc Trans **21**: 498S.

Sharp DC, Grubaugh WR, Weithenauer J, Davis SD and Wilcox CJ (1991). "Effects of Steroid Administration on Pituitary Luteinizing Hormone and Follicle-Stimulating Hormone in Ovariectomized Pony Mares in the Early Spring: Pituitary Responsiveness to Gonadotropin-Releasing Hormone and Pituitary Gonadotropin Content." Biology of Reproduction **44**: 983-990.

Sherwood NM, Sower SA, Marschak DR, Fraser BA and Brownstein MJ (1986). "Primary Structure of Gonadotropin-Releasing Hormone from Lamprey Brain." J Biol Chem **261**: 4812-4819.

Silverman AJ, Antunes L, Ferin M and Zimmerman EA (1977). "The Distribution of Luteinizing Hormone-Releasing Hormone in the Hypothalamus of the Rhesus Monkey. Light Microscopic Studies Using Immunoperoxidase Technique." Endocrinology **101**: 134-142.

Spiegel AM, Shenker A and Weinstein LS (1992). "Receptor-Effector Coupling by G Proteins: Implications for Normal and Abnormal Signal Transduction." Endocr Rev **13**: 536-565.

Stanislaus D, Ponder S, Ji TH and Conn PM (1998). "Gonadotropin-Releasing Hormone Receptor Couples to Multiple G Proteins in Rat Gonadotrophs and in Ggh3 Cells: Evidence from Palmitoylation and Overexpression of G Proteins." Biology of Reproduction **59**: 579-586.

Starling L, McIntosh JE and McIntosh RP (1988). "Estimating the Rate of Externalization of Gonadotrophin-Releasing Hormone Receptors in Ovine Anterior Pituitary Cells in Vitro." J Endocrinology **117**(1): 97-107.

Stojilkvic SS, Kukuljan M, Tomic M, Rojas E and Catt KJ (1993). "Mechanism of Agonist-Induced [Ca2+]I Oscillatioins in Pituitary Gonadotrophs." J Biol Chem **268**: 7713-7720.

Strauss SS, Chen CL, Kalra SP and Sharp DC (1979). "Localization of Gonadotropin Releasing Hormone in the Hypothalamus of Ovariectomized Pony Mares." J Reprod Fertil Suppl **27**: 123-129.

Stump W (1968). "Estradiol Concentrating Neurotopography in the Hypothalamus by Drymount Autoradiography." Science **162**: 1001-1003.

Sylvester PW, Uter HT and Meites J (1980). "Effects of Morphine and Naloxone on Phasic Release of Lh and Fsh." Proc Soc Exp Biol Mod **164**: 207-211.

Teresawa E, Krook C, Hei DL, Gearing M, Schultz NJ and Davis GA (1989). "Norepinephrine Is a Possible Neuortransmitter Stimulating Pulsatile Release of Luteinizing Hormone-Releasing Hormone in the Rhesus Monkey." Endocrinology **123**: 1808-1816.

Troskie BE, Hapgood J, Millar RP and Illing N (1998a). The Xenopus Laevis Gonadotropin-Releasing Hormone Receptor: Cloning and Characterization. Advances in Comparative Endocrinology. K. S. a. K. S., Monduzzi Editore, Italy.

Tsutsumi M, Laws SC, Rodic V and Sealfon SC (1995). "Translational Regulation of the Gonadotropin-Releasing Hormone Receptor in Alpha T3-1 Cells." Endocrinology **136**(3): 1128-1136.

Tsutsumi M, Laws SC and Sealfon SC (1993). "Homologous up-Regulation of the Gonadotropin-Releasing Hormone Receptor Alphat3-1 Cells Is Associated with Unchanged Receptor Messenger Rna (Mrna) Levels and Altered Mrna Activity." Molecular endocrinology **7**: 1625-1633.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, B and Sealfon SC (1992). "Cloning and Functional Expression of a Mouse Gonadotropin-Releasing Hormone Receptor." Molecular endocrinology **6**: 1163-1169.

Turner JE and Irvine CHG (1992). "The Effect of Various Gonadotropin-Releasing Hormone Regimes on Gonadotropins, Follicular Growth and Ovulation in Deeply Anestrous Mares." J Reprod Fertil Suppl **44**: 213-225.

Turzillo AM, Campion CE, Clay CM and Nett TM (1994). "Regulation of Gonadotropin-Releasing Hormone (Gnrh) Receptor Messenger Ribonucleic Acid and Gnrh Receptors During the Early Preovulatory Period in the Ewe." Endocrinology **135**(4): 1353-1358.

Turzillo AM, Clapper JA, Moss GE and Nett TM (1998a). "Regulation of Ovine Gnrh Receptor Gene Expression by Progesterone and Oestradiol." J Reprod Fertil **113**(2): 251-256.

Turzillo AM and Nett TM (1999). "Regulation of Gnrh Receptor Gene Expression in Sheep and Cattle." J Reprod Fertil Suppl **54**: 75-86.

Turzillo AM, Nolan TE and Nett TM (1998b). "Regulation of Gonadotropin-Releasing Hormone (Gnrh) Receptor Gene Expression in Sheep: Interaction of Gnrh and Estradiol." Endocrinology **139**(12): 4890-4894.

Valenca MM, Johnston CA, Ching M and Negro-Vilar A (1987). "Evidence for a Negative Ultrashort Loop Feedback Mechanism Operating on the Luteinizing Hormone-Releasing Hormone Neuronal System." Endocrinology **1221**(2256-2266).

Vizcarra JA, Wettemann RP, Braden TD, Turzillo AM and Nett TM (1997). "Effect of Gonadotropin-Releasing Hormone (Gnrh) Pulse Frequency on Serum and Pituitary Concentrations of Luteinizing Hormone and Follicle-Stimulating Hormone, Gnrh Receptors, Messenger Ribnucleic Acid for Gonadotropin Subunits in Cows." Endocrinology **138**(2): 594-601.

Vrecl M, Anderson L, Hanyaloglu A, McGregor AM, Groarke AD, Milligan G, Taylor PL and Eidne KA (1998). "Agonist-Induced Endocytosis and Recycling of the Gonadotropin-Releasing Hormone Receptor: Effect of Beta-Arrestin on Internalization Kinetics." Molecular endocrinology **12**: 1818-1829.

Watson N, Linder ME, Druey KM, Kehrl JH and Blumer KJ (1996). "Rgs Family Members: Gtpase-Activating Proteins for Heterotrimeric G-Protein Alpha-Subunits." Nature **383**: 172-175.

Weesner GD and Matteri RL (1994). "Rapid Communication: Nucleotide Sequence of Luteinizing Hormone- Releasing Hormone (Lhrh) Receptor Cdna in the Pig Pituitary." J Anim Sci **72**(7): 1911.

Weick RF (1978). "Induction of the Lh Surge by Intra-Hypothalamic Application of Estrogen in the Rhesus Monkey." Endocrinology **102**: 102:A23.

White BR, Duval DL, Mulvaney JM, Roberson MS and Clay CM (1999). "Homolgous Regulation of the Gonadotropin-Releasing Hormone Receptor Gene Is Partially Mediated by Protein Kinase C Activation of an Activator Protein-1 Element." Molecular endocrinology **4**: 566-577.

Wiley HS and Cunningham DD (1982). "The Endocytotic Rate Constant." The Journal of Biological Chemistry **257**(3): 4222-4229.

Willars GB, Heding A, Vrecl M, Sellar R, Blomenrohr M, Nahorski SR and Eidne KA (1999). "Lack of a C-Terminal Tail in the Mammalian Gonadotropin-Releasing Hormone Receptor Confers Resistance to Agonist-Dependent Phosphorylation and Rapid Desensitization." The Journal of Biological Chemistry **274**(42): 30146-30153.

Windle JJ, Weiner RI and Mellon PL (1990). "Cell Lines of the Pituitary Gonadotrope Lineage Derived by Target Oncogenesis in Transgenic Mice." Molecular endocrinology **4**: 597-603.

Wooge CH and Conn PM (1988). "Characterization of Calmodulin-Binding Components in the Pituitary Gonadotrope." Mol Cell Endocrinol **56**: 41-51.

Wu JC, Sealfon SC and Miller WL (1994). "Gonadal Hormones and Gonadotropin-Releasing Hormone (Gnrh) Alter Messenger Ribonucleic Acid Levels for Gnrh Receptors in Sheep." Endocrinology **119**: 1846-1850.

Xin JH, Cowie A, Lachance P and Hassell JA (1992). "Molecular Cloning and Characterization of Pea-3, a New Member of the Ets Oncogene Family That Is Differentially Expressed in Mouse Embryonic Cells." Genes Dev **6**: 1163-1169.

Xinwei L, Cornea A, Janovick JA and Conn PM (1998). "Visualization of Unoccupied and Occupied Gonadotropin-Releasing Hormone Receptors in Living Cells." Molecular and Cellular Endocrinology **146**: 27-37.

Yang-Feng TL, Seeburg PH and Fancke U (1986). "Human Luteinizing Hormone Releasing Hormone Gene (Lhrh) Is Located on Short Arm of Chromosone 8 (Region 8p11.2p.21)." Somatic Cell Mol Genet **12**: 95-100.

Zhou W and Sealfon SC (1994). "Structure of the Mouse Gonadotropin-Releasing Hormone Receptor Gene: Variant Transcripts Generated by Alternative Processing." DNA Cell Biol. **13**: 605-614.

BIOGRAPHICAL SKETCH

Michael Byron Porter was born January 31st of the year 1970 in Bogota, Colombia. For the first 7 years of his life, Michael remained in Bogota with his two sisters, Lisa and Lori, and his parents, Byron and Lilliana. In 1977, Michael and his family moved to the United States of America and settled in Miami, Florida, were Michael attended elementary as well as high school. In 1988, Michael enrolled at the University of Virginia in Charlottesville, Virginia. Four years later (1992), Michael received a Bachelor of Arts with a major in biology from the University of Virginia. In August of 1992, Michael enrolled at the University of Florida as a graduate student pursuing a Master of Science in the Animal Science Department. He completed his degree in 1994 and subsequently began his studies at University of Florida's College of Veterinary Medicine. While enrolled in veterinary school, Michael began the first joint DVM-PhD program at the University of Florida in January 1995. He graduated from veterinary school in May of 1998 and continued to work on his PhD. Michael has developed a keen interest in reproductive and medical physiology of horses and plans on pursuing these interests in an academic position.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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